Cheese Consumption and Human Microvascular Function

NCT03376555

September 21, 2021

PENNSTATE

HRP-592 - Protocol for Human Subject Research with Use of Test Article(s)

Protocol Title:

Cheese Consumption and Human Microvascular Function (IRB#4340)

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Version Date: 8/30/17

Clinicaltrials.gov Registration #:

1.0 Objectives

1.1 Study Objectives

Specific Aim 1. To characterize the vasoprotective effects of cheese on microvascular dysfunction induced by dose-dependent increases in dietary sodium in healthy human adults.
Hypothesis 1a: A high-cheese diet (6 servings/d x 7 d) preserves vasodilation responses to physiological (local heating) and pharmacological (acetylcholine infusion) stimuli in the cutaneous microvessels that are otherwise impaired by a high-sodium diet (5500 mg vs 1500 mg sodium).
Hypothesis 1b: The vasoprotective effects of cheese will be through nitric oxide (NO)- (endothelium-) dependent mechanisms.

Specific Aim 2. To identify the mechanisms by which cheese protects against dietary Na-induced oxidative stress in healthy human adults.
Hypothesis 2a: A high-cheese diet protects against oxidative stress in vessels by reducing the accumulation of superoxide radical that is otherwise increased by a high-sodium diet.
Hypothesis 2b: A high-cheese diet attenuates biomarkers of lipid peroxidation, protein oxidation, and inflammation that otherwise increase with increasing dietary sodium.

1.2 Primary Study Endpoints

Blood biomarkers as well as microcirculation and conduit vessel function will be measured to elucidate the mechanistic and functional effects of cheese on vascular health.

1.3 Secondary Study Endpoints

2.0 Background

2.1 Scientific Background and Gaps

Cardiovascular disease (CVD) is the leading cause of mortality in developed nations, with 40% of all deaths in the United States attributable to CVD. The annual health care burden of the treatment and management of CVD is greater than $350 billion and is projected to increase as our population ages[9, 10]. As such, identification of modifiable risk factors and non-pharmacological interventions are cornerstones of global CVD prevention. Increasing dairy intake is an emerging lifestyle factor that is associated with a decreased CVD risk[11, 12], yet the majority of Americans over the age of 50 do not meet the recommended dietary guidelines of 3 servings per day[13]. Interestingly, increased consumption of dairy products may inadvertently increase dietary sodium intake, as natural cheeses are often high in sodium. The American Heart Association recommends limiting daily sodium intake to 1500 mg Na/day for healthy individuals, and less for those at higher risk. Consequently, increasing dairy consumption, particularly in the form of cheese, may paradoxically hinder adherence to dietary sodium recommendations while mitigating CVD risk. It is currently unknown whether vasoprotective activities of dairy, provided as natural cheeses, protect against sodium-induced impairments in vascular function to lower CVD risk.

2.2 Previous Data

The positive impact of chronic dairy consumption on cardiovascular health, an area that we have been actively studying[14], has been demonstrated in many population-based studies[15, 16]. Increased total dairy intake is associated with decreases in global measures of vascular health and function including blood pressure [17], pulse wave velocity[8], arterial compliance [17], and arterial stiffness[16]. Long term dairy consumption is associated with lower blood pressure in healthy aged individuals[18], but its cardioprotective activities are also known to be mediated independent of any blood pressure-lowering effect[19], consistent with our co-investigator's controlled studies examining dairy consumption on postprandial vascular function[14, 20, 21]. Further, increased dairy consumption induces a modest but clinically significant reduction in blood pressure in those with previously elevated blood pressure (-4.0 mmHg SBP and -1.9mmHg DBP)[22]. The proposed mechanisms mediating these improvements in vascular function with increased dairy consumption are multifaceted and include angiotensin converting enzyme (ACE) inhibition[23-25], protection and enhancement of bioavailable NO[22, 26, 27], and anti-inflammatory and anti-oxidant properties[26, 28-30] of dairy proteins and micronutrients.

Dietary sodium intake is independently associated with elevations in arterial blood pressure[32] as well as increased cardiovascular morbidity and mortality[2]. Individual sodium excretion occurring with increased dietary intake of sodium >5.8 g/day is strongly associated with increased systolic and diastolic pressures of 10-11 mmHg and 6 mmHg, respectively. Moreover, this sodium-induced elevation in blood pressure increases with age[33]. High quality meta-analyses indicate that reducing sodium intake reduces blood pressure and the risk of stroke and fatal coronary heart disease[34].

Public health recommendations for reducing sodium intake are supported by physiological data on acute and chronic differences in sodium ingestion on endothelial and microvascular function in both animal[37] and human models[38]. Human studies demonstrate that sodium restriction (≤1.5 g/day) reverses age-associated endothelial dysfunction by increasing NO-dependent vasodilation and decreasing superoxide dismutase (SOD) expression[6]. Non-invasive measures of conduit artery endothelial function show that low dietary sodium intake is associated with enhanced flow-mediated vasodilation in middle aged and older adults[43]. Conversely, even short-term increases in dietary sodium impair flow-mediated vasodilation in conduit arteries of otherwise healthy young adults[38, 44, 45]. Germane to the present proposal, short term increases in dietary sodium (7 days) induce microvascular dysfunction in the cutaneous circulation[38] through increased oxidant stress mechanisms[7]. These sodium-induced vascular impairments are independent of changes in blood pressure, and suggest that acute sodium-induced alterations in vessel function are (1) endothelial in origin, and (2) primarily mediated by increases in oxidant stress via the generation of superoxide. The proposed sources of oxidant stress in the microvasculature, as well as pharmacological interventions that have been shown to be safe and efficacious for examination of these mechanisms *in vivo* in the human cutaneous microcirculation[46-48].

One putative mechanism by which dairy consumption may benefit vascular function is through the antioxidant properties of dairy proteins. Administration of dairy proteins in animal models reduces markers of inflammation such as TNF-α, soluble VCAM-1, and hsCRP; and attenuates measures of oxidative stress, including total antioxidant capacity [26, 28, 29].

In support of this hypothesis, our data demonstrate that *acute* (single-meal) cheese consumption is protective against sodium-induced impairments in NO-dependent vasodilation. We provided test foods, specifically cheddar cheese, pretzels, or soy cheese, that were matched for sodium (560 mg or 1120 mg) prior to evaluating sensitive measures of NO-dependent vasodilation. We demonstrated that the acute consumption of cheese (one meal) prevented sodium-induced impairment in endothelial function[49] (while there was no difference in plasma sodium between treatments containing 560mg sodium (cheddar: 133.8±1.8; pretzel: 135.2±1.0; soy: 134.4±1.8 $mmol \cdot L^{-1}$) or 1120mg sodium (cheddar: 135.2±0.5; pretzel 136.1±1.8 $mmol \cdot L^{-1}$). Furthermore, acute localized administration of the antioxidant ascorbate normalized NO-dependent vasodilation following non-dairy sodium ingestion, but had no effect on NO-dependent vasodilation after ingesting natural cheese. These data strongly suggest that non-Na components of natural cheese protect against acute sodium-induced endothelial dysfunction in the microvasculature of healthy middle-aged adults. This protective effect appears to be mediated by the antioxidant properties of natural cheese, which are potentially mediated through the known antioxidant activities of dairy proteins[19, 50]. Because there were no differences in plasma sodium concentrations and the timing of the functional assessment of NO-dependent vasodilation was at the expected peak plasma concentrations of dairy proteins[14], this effect is not likely related to differences in gastric emptying rates. Collectively, these data suggest that the micro and macro nutrients in natural cheeses may mitigate sodium-induced vessel dysfunction through anti-oxidant mechanisms.

2.3 Study Rationale

Epidemiological studies suggest that a high-sodium diet is associated with increased risk of cardiovascular disease (CVD)[1, 2] whereas high dairy intakes, which can be high in sodium, improves cardiovascular health across the lifespan[3-5]. Controlled studies show that a high sodium intake induces endothelial dysfunction by reducing nitric oxide (NO)-dependent vasodilation (VD)[6, 7]. Although dairy provides vasoprotective effects on the vasculature that are, in part, mediated through improving NO-dependent vasodilation[8], it remains unclear if these benefits of dairy, especially those potentially attributed to natural cheeses, occur independent of sodium intake. Over the past funding cycle, our laboratory has advanced an understanding by showing that acute (one meal) ingestion of natural cheese protects against sodium-induced reductions in NO-dependent vasodilation. Our collaborative expertise in vascular biology, cellular inflammation and oxidative stress, and controlled-feeding trials is ideally positioned to conduct seminal studies demonstrating that cheese, despite contributing to sodium intakes, protects against vascular dysfunction and the mechanisms by which this occurs. Specifically, the present study utilizes highly-sensitive *in vivo* microvascular methodologies, coupled with robust measures of conduit vessel function, to define vasoprotective oxidant stress-mediated mechanisms of cheese (6 servings/d) in a 2 x 2 factorial design of controlled, 7 d dietary interventions using low-sodium (1500 mg/d) and high-sodium (5500 mg/d) diets as a relevant clinical model of sodium-induced vessel dysfunction.

3.0 Inclusion and Exclusion Criteria

3.1 Inclusion Criteria

Men and women (55-75 years)
Apparently healthy
Normotensive through mildly hypertensive (pre-hypertensive-Stage 1; <140/90 mmHg) blood pressures
    Seated systolic pressure 120-140 mmHg
    Seated diastolic pressure 70-90 mmHg.
Normoglycemic (HbA1C <5.7%)

3.2 Exclusion Criteria

Taking pharmacotherapy that alters peripheral vascular control
Pregnancy
Breastfeeding
Females taking contraceptives (pills, patches, shots, etc.) or hormone replacement therapy
Taking illicit and/or recreational drugs
Use of nicotine containing-products (e.g. smoking, chewing tobacco, etc.)
Known allergy to latex or investigative substances

3.3 Early Withdrawal of Subjects

3.3.1 Criteria for removal from study

Participants may withdraw at any time. We may end the participant's role in the study without her/his consent if her/his seated blood pressure becomes higher than that specified under the inclusion criteria. Also, we may end the participant's role if we determine that her/his health or behavior adversely affects the study or increases the risks beyond those approved by the Institutional Review Board and agreed upon by her/him in the informed consent.

3.3.2 Follow-up for withdrawn subjects

If the participants withdrew immediately during or after an experiment, we contact the participants within 5 days.

4.0 Recruitment Methods

4.1 Identification of subjects

We recruit subjects from Centre County, PA and surrounding regions utilizing CTSI resources at University Park. Also, we advertise for subjects (see below). Interested persons contact us. We also avail ourselves of lists of potential subjects maintained by the CRC and our lab. People on these lists have screened for other studies and indicated that they wished to be maintained on a list of potential subjects to be contacted in the event that they may qualify for additional studies.

4.2 Recruitment process

We advertise for subjects. Interested persons contact us. We discuss the study's purpose and protocol as well as the qualifications for the study with the potential subject. We discuss the informed consent with them. We invite potential subjects to tour the lab, and ask questions.

4.3 Recruitment materials

Newspaper/magazine ads
Letters/Emails to potential participants
Flyers/posters
Brochures
Web Sites The website and initial screening form collect the same basic pre-screening information.
Listserv
Script - Verbal (i.e., telephone, face-to-face, classroom)
Note: We advertise through various listservs on and off campus that target under-represented groups (e.g. FOBA) as well as listservs belonging groups (e.g. Elks) and others as we become aware of them. We have a recruiting website through the Kinesiology department that we have used for most of our studies. We use the information from the uploaded ad and/or the phone script in other forms of recruitment (e.g. emails, listservs).

4.4 Eligibility/screening of subjects

People may contact us. We conduct a basic interview (see uploaded initial screening form) with the potential subjects and discuss the study with them. See "Study Design and Procedures" for screening information.

5.0 Consent Process and Documentation

5.1 Consent Process

5.1.1 Obtaining Informed Consent

5.1.1.1 Timing and Location of Consent

The informed consent is signed before screening procedures have begun when the subject reports to Noll Lab or the CRC. If subjects are not able to understand the protocol and instructions for any reason, written or verbal, they are not included in the study. Subjects are informed throughout the consenting, screening, and conduction of the study that they may discontinue their participation at any time.

5.1.1.2 Coercion or Undue Influence during Consent

Subjects are informed throughout the consenting, screening, and conduction of the study that their participation is voluntary, and they may discontinue their participation at any time. It is possible that a person enrolled in the study could be a student or employee. We tell the person that participation in the study is voluntary and no aspect of their

participation or non-participation has an effect on their class or grade, employment or salary, respectively. The person conducting the screening and consenting is not a professor from any of the student's classes or the employee's supervisor. We inform patients that participation, non-participation, or withdrawal from the study does not affect the quality of their medical care, payment or enrollment in any health plans, or affect eligibility for benefits.

5.1.2 Waiver or alteration of the informed consent requirement

NA

5.2 Consent Documentation

5.2.1 Written Documentation of Consent

When a potential participant reports to Noll Lab or CRC, she/he signs the informed consent before screening procedures begin. After the participant signs the consent, we give the participant a photocopy.

5.2.2 Waiver of Documentation of Consent (Implied consent, Verbal consent, etc.) NA

5.3 Consent – Other Considerations

5.3.1 Non-English Speaking Subjects NA

5.3.2 Cognitively Impaired Adults NA

5.3.2.1 Capability of Providing Consent

5.3.2.2 Adults Unable To Consent

5.3.2.3 Assent of Adults Unable to Consent

5.3.3 Subjects who are not yet adults (infants, children, teenagers) NA

5.3.3.1 Parental Permission

5.3.3.2 Assent of subjects who are not yet adults

6.0 HIPAA Research Authorization and/or Waiver or Alteration of Authorization NA

6.1 Authorization and/or Waiver or Alteration of Authorization for the Uses and Disclosures of PHI
Check all that apply:

X **Not applicable, no identifiable protected health information (PHI) is accessed, used or disclosed in this study.** *[Mark all parts of sections 6.2 and 6.3 as not applicable]*

☐ **Authorization will be obtained and documented as part of the consent process.** *[If this is the only box checked, mark sections 6.2 and 6.3 as not applicable]*

☐ **Partial waiver is requested for recruitment purposes only (Check this box if patients' medical records will be accessed to determine eligibility before consent/authorization has been obtained).** *[Complete all parts of sections 6.2 and 6.3]*

☐ **Full waiver is requested for entire research study (e.g., medical record review studies).** *[Complete all parts of sections 6.2 and 6.3]*

☐ **Alteration is requested to waive requirement for written documentation of authorization (verbal authorization will be obtained).** *[Complete all parts of sections 6.2 and 6.3]*

6.2 Waiver or Alteration of Authorization for the Uses and Disclosures of PHI

6.2.1 Access, use or disclosure of PHI representing no more than a minimal risk to the privacy of the individual

6.2.1.1 Plan to protect PHI from improper use or disclosure

6.2.1.2 Plan to destroy identifiers or a justification for retaining identifiers

6.2.2 Explanation for why the research could not practicably be conducted without access to and use of PHI

6.2.3 Explanation for why the research could not practicably be conducted without the waiver or alteration of authorization

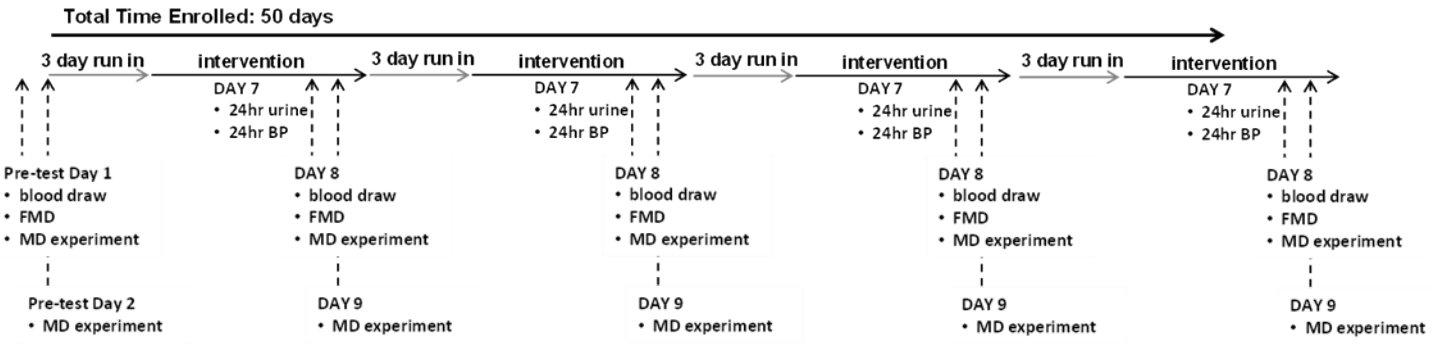
6.3 Waiver or alteration of authorization statements of agreement

7.0 Study Design and Procedures

7.1 Study Design

This study's goal is to determine the vasoprotective effects of natural cheese, provided as natural cheddar cheese, on sodium-induced impairments in microvascular function in an otherwise healthy aged population. Our research team will

conduct a 4-arm crossover study in which participants will receive a high-cheese diet (6 servings/d) or one devoid of cheese for 7 days in the context of a low-sodium (1500 mg) or high-sodium (5500 mg) basal diet. A 7-day controlled feeding regimen of altered sodium consumption is consistent with our studies showing efficacy in modulating cutaneous microvascular function in humans[7]. Our approach will utilize sensitive methodologies specific to the microcirculation to mechanistically examine the role of oxidant stress in sodium-induced human NO- mediated vascular endothelial dysfunction and the potential mitigating effects of natural cheese. These measures: (1) are accurate and quantifiable, (2) are highly reliable, (3) represent the earliest clinical indicator of vessel dysfunction, (4) are predictive of future impairments in conduit vessel function, and (5) enable underlying mechanisms to be reliably explored. Blood biomarkers and conduit vessel function will be measured to elucidate the mechanistic and functional effects of cheese on vascular health. (See Appendix I)



7.2 Study Procedures

(See section 7.4.1 for names and descriptions of investigative substances)

Overview: Microdialysis (MD) is a procedure in which a thin tube of membrane that mimics the capillary blood vessel is implanted in tissue. As a physiological saline perfuses the membrane, there is bi-directional exchange of molecules between the perfusing saline and the fluid bathing the tissue surrounding the membrane. However, the membrane prohibits the exchange of large molecules such as proteins. Substances of interest may be added to the perfusing saline and thus delivered into the surrounding tissues with no systemic effects.

All experiments occur in the morning.
Subjects fast for 8 hours prior to experiments so the data reflect the long term effects of the subject's diet over the previous 7 days rather than the short term effects of meals consumed just prior to experiments.

A. Screening

1. The participants drink only water and do not eat for 12 hours before the screening.
2. The research nurse conducts the screening that includes pregnancy test for women of childbearing-age, heart rate (HR), blood pressure (BP), height, waist circumference, weight, health history, and standard venipuncture to obtain blood (50 ml, <4 Tbsp) for complete blood count (CBC), chemistry analysis, lipid profile, and other substances of interest (i.e. Interleukin Panel).
3. If participant takes thyroid medication, the nurse obtains thyroid stimulating hormone (TSH) level from participant. If TSH level is not available or and has not been measured within 6 months, TSH is measured from blood sample (3.5 ml; 0.2 Tbsp).
4. The researchers do not perform genetic analyses on the blood nor look for presence of disease (e.g. HIV).
5. Subjects undergo ambulatory 24 hour blood pressure monitoring.

If the research nurse is unavailable for an extended period, the Clinical Research Center CRC staff performs her screening tasks. The CRC uses their admission form to admit potential participants for screening at the CRC.

Participants meet with a registered dietitian (Amy Ciccarella, R.D.; PSU CTRC dietitian) to determine their eucaloric energy requirements and identify food preferences. The interview includes surveying the subject's physical activity over the previous 7 days. (This meeting can occur on a day separate from that of the screening.)

B. Baseline Experiments (Separate Days, the order of the local heating and acetylcholine dose response experiments is randomized)

1. Baseline nutrient assessment: Subjects complete a 3-day food record that is evaluated using the Nutrition Data System for Research (NDSR) dietary analysis software.
2. Preparation for experiments
   a. We give printed and verbal instructions outlining what the subjects need to do before they come to the lab.
   b. Subjects continue with their normal diet before and during the days of the baseline experiments except:
      i. They refrain from consuming alcohol, niacin supplements, and fish oils for 48 hours, and caffeine (ex. coffee, tea, Coca Cola, chocolate) for 12 hours before the experiment.
      ii. They fast for 8 hours prior to each experiment.
      iii. They drink only water for 8 hours prior to each experiment.
   c. On the day of the experiment,
      i. Subjects refrain from activity that causes them to exert themselves more during a leisurely walk.
      ii. We measure HR, BP, and oral temperature. Women who are not postmenopausal undergo a urine pregnancy test if they have not had one within 2 weeks.
      iii. Microdialysis probe insertion: The subject washes the ventral forearm with antimicrobial soap. We place a tight band around the arm so we can visualize veins. For each MD site, we make pairs of pen-marks on the arm 2.5 cm (1 inch) apart and away from veins. We remove the tight band. The MD tubing enters and exits the skin at the marks. We clean the arm with povidone iodine and alcohol. We place an ice bag on the arm for 5 minutes to numb the skin. Then we insert a thin needle into the skin at each entry mark. The needle's tip travels between the layers of skin for 2.5 cm (1 inch) and leaves the skin at the matching exit mark. We thread the MD tubing through the needle. Next, we withdraw the needle leaving the tubing in the skin. Any redness of the skin subsides in about 60 – 120 minutes.
      iv. We attach 3 ECG leads to measure heart rate, and place a cuff on the upper arm sans the MD probes to measure blood pressure. We place a local skin heater and laser Doppler probe over each MD site. We measure heart rate, local temperature, and skin blood flow (SkBF) throughout the MD experiments. We also measure blood pressure every 5-7 minutes (brachial osculation and/or Cardiocap).

3. Baseline Experiments – "Day 1"
   a. FMD

      FMD assesses conduit vessel endothelial function. We place a BP cuff on a forearm, gel on the upper arm just above the elbow, and a Doppler ultrasound probe on the gel. The ultrasound measures vessel size and blood velocity. After a 3-minute "resting" measurement, the cuff inflates for 5 minutes to occlude forearm BF. After the cuff deflates, we perform a second reading. We may repeat this measure several times.

      Sublingual nitroglycerin: This procedure assesses vascular smooth muscle function. A nurse administers the sublingual nitroglycerine and is present throughout the procedure. The subject is supine on a medical bed or recliner. We apply a blood pressure / heart rate monitor (Cardiocap). We may take manual blood pressures. Also, we perform ultrasonography at the brachial artery at the elbow during the procedure. We place a 0.4 mg nitroglycerin tablet under the subject's tongue. The subject closes the mouth immediately after we insert the tablet. The tablet dissolves in 15-90 seconds. The subject refrains from swallowing until the tablet dissolves. Nitroglycerin causes blood vessels to dilate. The effects last for 5-10 minutes. The subject remains supine for 20 minutes following the nitroglycerin administration. The subject remains in the lab until 20 minutes after administration of the nitroglycerin. If the subject has an exceptional reaction to the nitroglycerin (e.g. drop in blood pressure with longer duration), we monitor the subject for up to 60 minutes following nitroglycerin administration.

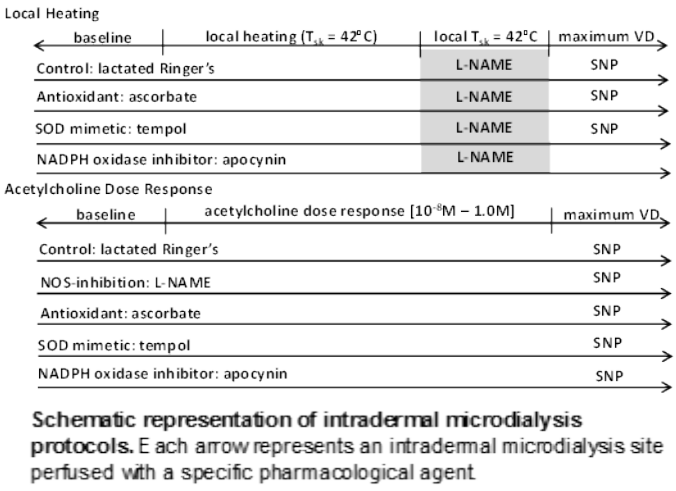
   b. MD: Local Heating

We prepare 4 MD sites:

Probe 1. Lactated Ringer's only (control)
Probe 2. Lactated Ringer's + Ascorbate
Probe 3. Lactated Ringer's + Tempol
Probe 4. Lactated Ringer's + Apocyanin

Initially, we set the local heaters to 34°C (93°F), and only Ringer's perfuses each probe. We collect baseline data for 20 minutes, then we add the investigative substances to the probes. After a second 20-minute baseline, we increase the temperature of the local heaters 0.5°C/5 sec to a target local skin temperature of 42°C (107.6°F). We clamp the local temperature at 42°C (107.6°F). When the SkBF stabilizes, we add L-NAME to all probes to allow for the quantification of NO-dependent vasodilation. After the SkBF stabilizes (~40 minutes), we switch perfusates at all MD sites to Ringer's + sodium nitroprusside (SNP) for about 30 minutes. Heating and SNP perfusion causes maximum vasodilation.

c. Then the experiment is over, and we remove the MD tubing from the skin and place sterile bandages over the sites. If the subject desires, we can also place a bag of ice on the site for 10 minutes to reduce any bruising that may occur. We measure blood pressure and heart rate before the subject departs.



Schematic representation of intradermal microdialysis protocols. Each arrow represents an intradermal microdialysis site perfused with a specific pharmacological agent

4. Baseline Experiments – "Day 2"
   a. MD: Acetylcholine (ACh) Dose Response
   We prepare 5 MD sites:

   Probe 1. Lactated Ringer's only (control)
   Probe 2. Lactated Ringer's + LNAME
   Probe 3. Lactated Ringer's + Ascorbate
   Probe 4. Lactated Ringer's + Tempol
   Probe 5. Lactated Ringer's + Apocyanin

   We clamp the temperatures of the local heaters at 34°C (93°F). When the experiment begins, the subject rests for a 20-minute baseline. When SkBF readings are stable, we add the test substances to probes 3, 4, and 5 as indicated above for about 60 minutes. After 30 minutes, we add LNAME to probe 2. During this time, we obtain a second baseline for about 20 minutes. When the SkBF is stable, we add the first concentration of ACh to the perfusate at each probe. Each site receives 11 increasing concentrations of ACh (10^-8M to 10^-1M) each perfused for 5 minutes. As SkBF stabilizes with the addition of each concentration of ACh, we proceed to the next concentration. After the last concentration of ACh, we switch perfusates at all sites to Ringer's + SNP for about 30 minutes. Heating and SNP perfusion causes maximum vasodilation.

   b. Then the experiment is over, and we remove the MD tubing from the skin and place sterile bandages over the sites. If the subject desires, we can also place a bag of ice on the site for 10 minutes to reduce any bruising that may occur. We measure blood pressure and heart rate before the subject departs.

5. Subjects wait at least 3 days before proceeding with the Controlled Feeding protocols.

C. <u>Controlled Feeding Experiments</u> (the order of the local heating and acetylcholine dose response experiments is randomized)

1. Feeding Protocol

Following baseline experiments outlined above, participants are enrolled in a randomized, cross-over protocol involving 4 controlled feeding periods, each 8 days in duration, (7 days prior to subsequent experiments plus the day

of first experiment). Subjects maintain the assigned feeding protocol through the 2 days of experiments. The feeding protocols are:

low sodium diet devoid of dairy products (L-Na)
low sodium diet containing cheese (L-Na+C)
high sodium diet devoid of dairy products (H-Na)
high sodium diet containing cheese (H-Na+C)

Three-day rotating menus are developed by Richard Bruno, PhD, RD (Ohio State University) for each 8-day controlled feeding period to provide diets low or high in sodium (1500 vs 5500 mg) in combination with a cheese-free or cheese-containing diet (0 vs. 6 servings/d).

All foods and beverages are prepared by our highly trained staff in the PSU Clinical Translational Research Center (CTRC) metabolic kitchen, packaged appropriately, and provided to participants for each 8-day feeding period. Participants eat the prescribed diet at home, but are required to consume only those foods provided to them and to return any uneaten portions to accurately quantify food and beverage consumption. To ensure compliance and maintain effective rapport, participants visit the study center every 1-2 days to pick up food and drop off containers from the preceding 24-48 hours. Food intake is determined by weighing uneaten portions, and nutrient intakes calculated by a dietitian (Ms. Ciccarella) using NDSR software.

2. Preparation for experiments
   a. We give printed and verbal instructions outlining what the subjects need to do during the 8-day dietary intervention and before they come to the lab for experiments.
   b. Subjects consume only the assigned diet for eight days. We encourage subjects to eat all food we give to them especially the cheese.
   c. Subjects fill out a daily questionnaire regarding the food/drink they consumed over the previous 24 hours.
   d. They visit the study center every 1-2 days to pick up food and drop off containers.
   e. While on the diet, subjects refrain from consuming alcohol, antioxidants (i.e. vitamin C) and drugs with salt (e.g. antacids).
   f. On day seven of each dietary intervention, the subjects collect a 24-hour urine (sodium excretion) and undergo 24-hour blood pressure monitoring. Subjects exhibiting a significant increase in blood pressure following the high sodium condition are classified as salt-sensitive and excluded.
   g. Subjects refrain from consuming caffeine (ex. coffee, tea, Coca Cola, chocolate) for 12 hours before the experiment.
   h. They fast for 8 hours prior to each experiment.
   i. They drink only water for 8 hours prior to each experiment.
   j.
   k. On the day of the experiment,
      i. We measure HR and BP. Women who are not postmenopausal undergo a urine pregnancy test if they have not had one within 2 weeks.
      ii. Microdialysis probe insertion (described above)
      iii. We attach 3 ECG leads to measure heart rate, and place a cuff on the upper arm sans the MD probes to measure blood pressure. We place a local skin heater and laser Doppler probe over each MD site. We measure heart rate, local temperature, and skin blood flow (SkBF) throughout the MD experiments. We also measure blood pressure every 5-7 minutes (brachial osculation and/or Cardiocap).

3. Experiments “Day 8”
   a. Subjects undergo 30 ml (2 Tbsp) blood draw to measure plasma oxidant stress and inflammatory markers.
   b. FMD (described above)
   c. MD Experiment - Local Heating (or Acetylcholine Dose Response) described above
   d. After the experiment, subjects resume the controlled feeding protocol. They eat as much of the food suppled for Day 8 as they can.

4. Experiments "Day 9"
   a. MD Experiment - Acetylcholine Dose Response (or Local Heating) described above.
   b. Subjects resume normal diet.

5. Subjects wait at least 3 days before beginning the next Controlled Feeding period. They undergo the 7-day Controlled Feeding periods followed by 2 days of experiments for each of the 4 feeding protocols. Subjects repeat the 3-day food record during one of the washout periods.

D. Equipment:

Laser Doppler Flowmetry: The Laser Doppler Flowmeter (Moor Instruments, Inc.) non-invasively provides a qualitative measure of skin blood flow to a depth of about 1 mm in the skin using a weak laser light. This measure is a dimensionless value called "flux" that reflects the speed and number of blood cells moving through the microvasculature in an area of skin. The flowmeter continuously measures skin blood flow using a fiber optic probes that fit into holders taped to the skin. approved by the FDA

Blood Pressure, ECG (Heart rate): The CardioCap 5 (General Electric – GE) critical care monitor measures blood pressure via a cuff inflated every 5-7 minutes on the upper arm and heart rate via 3-lead ECG probes taped to the skin of the chest. approved by the FDA

Flow Mediated Dilation (FMD): FMD measures the health of blood vessels. The researchers place a blood pressure cuff around the forearm. They put gel on the upper arm just above the elbow. Then they place a Doppler ultrasound probe on the gel. The ultrasound makes sound waves to measure the size of blood vessels and the speed of the blood. They make a "resting" measurement before they inflate the cuff. Then they inflate the cuff for 5 minutes to stop blood flowing to and from the forearm. After they deflate the cuff, they perform a second reading for 3 minutes. ATL HDI 5000 SonoCT (Ultrasound) FDA 510 (k) approved by the FDA

7.3 Duration of Participation

Screening (1 Visit)	less than 1.5 hour
FMD/MD Experiments (5 Visits)	less than 7 hours each
MD Experiments (5 Visits)	5 hours
Obtain Food, 24-hour BP monitor & urine container, etc. (~14 Visits)	about 15 minutes each

Total: ~75 Hours (~25 visits; It could take about 8 weeks to complete the study.)

7.4 Test Article(s) (Study Drug(s) and/or Study Device(s))

7.4.1 Description

Devices: See "7.2 Study Procedures"

Acetylcholine ACh Form: Powder

Role in Current Study: Local heating combined with the local perfusion of endothelial agonist, acetylcholine, specifically examines the attenuated endothelium-dependent vasodilation (eNOS-derived NO) associated with aging or vascular pathologies.

Apocynin Form: Powder

Role in Current Study: Apocynin inhibits NADPH oxidase, which is a primary source of superoxide production within the vasculature.

L-Ascorbate Vitamin C Form: Powder

Role in Current Study: L-ascorbate stabilizes norepinephrine in solution. A small amount will be delivered by MD to a nickel-sized are of skin.

Lactated Ringer's Ringer's Form: Liquid
Role in current study: Lactated Ringer's acts as the vehicle for the other research substances and as a flush.

LNAME N^G-nitro-L-arginine methyl ester Form: Powder
Role in current study: A nitric oxide synthase inhibitor. L-NAME is an analog to the amino acid, L-arginine. L-NAME is a non-specific inhibitor for nitric oxide synthases, thereby inhibiting the production of nitric oxide that causes vasodilation. We deliver small doses of LNAME to a nickel-sized area of the skin.

Sodium Nitroprusside SNP Form: Powder
Role in Current Study: SNP, acting an NO donor, dilates blood vessels maximally thereby achieving maximal skin blood flow (SkBF). Maximal SkBF is a reference point for other measures of skin blood flow.

Tempol Form: Powder
Role in Current Study: Membrane-permeable scavenger of free-radicals. Tempol scavenges superoxide in vivo and reduces oxidant stress, acting as a superoxide dismutase mimetic. Tempol increases NO bioavailability in the vasculature and improves endothelial function. MD delivers a very small amount to the blood vessels in a nickel-sized area of skin.

7.4.2 Treatment Regimen

Investigative Substances: Microdialysis
The following is a table of the research agents used with intradermal microdialysis. Based upon research literature, we use 14% for calculating maximum delivery of the research agents for all IND applications for our MD studies.

Research Agent	14% delivery (mg)
ACh	0.025
Apocynin	0.0005
Ascorbate	0.057
L-NAME	0.096
SNP	0.070
Tempol	0.00006

7.4.3 Method for Assigning Subject to Treatment Groups

NA

7.4.4 Subject Compliance Monitoring

Participants are to consume only those foods provided to them and to return any uneaten portions to accurately quantify food and beverage consumption. To ensure compliance and maintain effective rapport, participants visit the study center every 1-2 days to pick up food and drop off containers from the preceding 24-48 hours. Food intake is determined by weighing uneaten portions, and nutrient intakes calculated by a dietitian.

7.4.5 Blinding of the Test Article

NA

7.4.6 Receiving, Storage, Dispensing and Return

7.4.6.1 Receipt of Test Article

The containers of the substances are dated upon receipt and when opened.

Research Agent	Source(s)
ACh	USP
Apocynin	Sigma
Ascorbate	Sigma
L-NAME	EMD, Tocris
Lactated Ringer's	VWR, McKesson, Moore Medical
SNP	USP
Tempol	Calbiochem

From uploaded Physician Oversight SOP:
The investigative agents are purchased by the lab members from reputable sources as indicated in the IRB and the FDA IND applications and in accordance with the standards and guidelines imposed by the FDA. A license from the CRC

physician, the nurse manager, or the overseeing physician is filed with vendors (e.g. VWR , Owens and Minor) requiring the documentation for the purchase of some investigative agents (e.g. Lactated Ringer's) . The investigative agents are shipped to the Noll Lab or picked up from the pharmacy by lab personnel. Copies of the prescriptions and orders are maintained in the laboratory's files.

7.4.6.2 Storage

Our procedures regarding the handling and use of the drugs have been extensively examined by the FDA and approved. The temperatures are recorded daily during weekdays. The temperature of the room is also monitored by the Central Control System of the Environmental Systems at PSU. The drugs used with microdialysis in their solid form are stored under environmental conditions according to manufacturer's instructions in cabinets, refrigerators, or freezers located in Room 224A or 228 Noll Laboratory. Opened drugs in their solid form are discarded after one year. The rooms are locked when unoccupied. General Note: The chemicals used for microdialysis in this study are scientific tools that are not FDA-approved, dispensable drugs, and do not have expiration dates. We purchase most of these chemicals in solid form. The chemicals are stable for years when stored according to manufacturer’s instructions. Nonetheless, we purchase most of the chemicals in small vials that we typically exhaust within one to several weeks. Once mixed, we use stock solutions of the drugs within a week if stored in the refrigerator. We use solutions within 6 months if they are frozen. Final dilutions of solutions prepared for an experiment are used within a couple of minutes or hours of preparation.

7.4.6.3 Preparation and Dispensing

None of the microdialysis drugs are dispensed to the participant; rather we use them in the experiment, as outlined in the Standard Operating Procedures (Phys Ovrsght SOP). The physician providing oversight approves the laboratory standard operating procedure for the obtainment, preparation, and administration of all investigational agents for MD and other procedures used in experiments.

From uploaded Physician Oversight SOP:
Investigative Substances used with Microdialysis
The following procedures have been examined and approved by the FDA.
Trained lab personnel prepare the perfusates in accordance with the procedure described in the IRB and FDA IND applications. When mixing the perfusates, the experienced technician washes the hands, wears protection (e.g. gloves , lab coat), and uses glassware that has been washed with cleaner (designed for use with healthcare instruments , pharmaceutical process equipment , tissue culture apparatus , etc.), and rinsed multiple times in tap and then doubly-distilled water and air-dried. Most of the investigational agents are obtained in ultra-pure solid form. The solid investigational agents are weighed on a microbalance and then mixed with sterile pharmaceutical-grade Lactated Ringer's solution to the desired concentration. The solution is drawn into the syringe through a 0.2 µm filter. Prior to injecting a solution into or withdrawing solution from a sterile container through a sterile hypodermic needle, the stopper of the sterile container is cleaned thoroughly with alcohol. Most perfusates are used within minutes or hours after preparation. Stock solutions are made for some investigational agents. The stock solutions are drawn into sterile 1-cc syringes through 0.2 µm filters for storage. The sterile stock solutions are labeled accordingly (e.g. content, date), and protected from light. Stock solutions are not used beyond 1 week (refrigerated) and 6 months (frozen) after mixing. Stock solutions are diluted with sterile Lactated Ringer's when preparing the perfusate. The final dilutions of the perfusates are drawn into sterile syringes through 0.2 µm filters within minutes or hours of their use in the experiment. The protocol that includes the administration of the investigative substances has been examined and approved by the FDA.
The administration of the investigative substances is performed by the trained and approved lab personnel as described in the protocol included in the IRB and FDA IND applications. The overseeing physician has observed and approved the microdialysis technique as performed by Drs. Anna Stanhewicz and Lacy Alexander. The lab personnel performing microdialysis have been trained and approved to perform the technique by the Dr. Lacy Alexander, approved by the overseeing physician, and approved by the IRB. Letters of approval from the overseeing physician for each member of the lab who performs microdialysis in the researcher project have been submitted to the IRB.

7.4.6.4 Return or Destruction of the Test Article

Usually, investigative substances are consumed in the experiments. Investigative substances not consumed by the experiments are disposed of in accordance with the policies of Environmental Health and Safety of Penn State. Some substances (e.g. protein or peptide-based) are autoclaved prior to disposal.

7.4.6.5 Prior and Concomitant Therapy

NA

8.0 Subject Numbers and Statistical Plan

8.1 Number of Subjects

Number of subjects to be enrolled: 25
Number of subject needed to complete study: 15

8.2 Sample size determination

Based on our previously published data 46, 115, 128, and data from our current cheese intervention study, we calculate that 14 subjects per group will be necessary to physiologically relevant differences between dietary interventions of at least 15%; (ANOVA, power=0.80, α=0.05).

Our published data examining within-group differences between microdialysis drug treatments (using similar localized microdialysis drug treatments) suggest that within groups with compromised vasodilatory function, sample sizes of 8 and 7 subjects per group would be sufficient to detect a meaningful difference (effect size of 15%) between microdialysis treatment sites examining particular pathways (1 sample t-test, e.g., NO-dependent dilation). Our data specific to dietary cheese and sodium interventions indicates that 8 subjects are necessary find a meaningful physiological difference.

We suggest testing 15 subjects through all of the dietary interventions. We would plan to perform preliminary analyses after testing 8 subjects to reach our desired statistical power for detecting within group differences between microdialysis sites.

8.3 Statistical methods

We use within-person experimental design and associated repeated measures ANOVA tests with planned contrasts for our primary functional outcomes. We use multivariate regression analysis with possible ANCOVA to determine which measures of oxidative stress and antioxidant status are correlated with our functional findings. For all experiments, we accept significance at $p<0.05$. We adjust P-values and 95% confidence intervals for the mean difference estimates using Tukey's multiple comparison procedure to account for post-hoc multiple comparison testing and ensure a familywise type I error rate less than 5%. We use residual plots to check modeling assumptions, specifically examining them for nonconstant variances and nonnormality. We analyze data using SAS PROC MIXED, a flexible analytic tool that accommodates a wide variety of analysis of variance and regression models and nested and incomplete data structures.

9.0 Confidentiality, Privacy and Data Management

9.1 Confidentiality

9.1.1 Identifiers associated with data and/or specimens

Most of the data are coded and do not contain personal identifying information. Documents allowing identification of participants do not leave our labs and are only available to authorized persons. The names and contact information that potential subjects submit via the secure Qualtrics website is copied to a password-protected lab computer. The subjects' information is deleted immediately from the Qualtrics website after being copied. Only authorized personnel may access the lab computer. Data forms containing identifiable information are shredded when no longer needed (within 6 years after publication of results).

9.1.1.1 Use of Codes, Master List

We keep data in the laboratory in locked cabinets, the password-protected folder on the secured PSU server, and on password-protected computers maintained in a locked room. Only authorized personnel have access. Coded data shared with unauthorized persons cannot be traced to individuals. The list linking code numbers to participants is not

shared with unauthorized persons and destroyed when project is completed and published. The code is destroyed within 5 years of publication of the data.

9.1.2 Storage of Data and/or Specimens

We maintain data in the laboratory in locked cabinets and on password-protected computers located in rooms that are locked when unoccupied. Most of the data are coded and do not contain personal identifying information. Documents allowing identification of participants do not leave the investigator's labs and are only available to authorized persons. Coded data shared with unauthorized persons cannot be traced to individuals. We store any list linking the code to participants' identity in locked cabinets and on password-protected computers located in rooms that are locked when unoccupied. Potential participants may submit pre-screening questionnaires via the investigator's website / Qualtrics (commercial survey website). All web traffic to and from the Qualtrics application is done via a Secure Socket Layer (SSL) that encrypts the data in transmission. Qualtrics deletes data from their servers within 24 hours after the user deletes it from the website. Hard copy data forms containing identifiable information are shredded when no longer needed (within 5 years after publication of results). Screening data from subjects who are not accepted into the study are shredded when the project ends. Subjects may give permission to have their contact information retained in the investigator's secured files if they wish to be considered for participation in future studies. After we complete the study, we remove all identifiers from the study's data and store the data indefinitely. Individual data may be used without identifying the subject to illustrate representative responses.
Biological specimens are stored at University Park and Quest Labs, (Chantilly, VA), Ohio State University (Columbus, OH), and Georgia Health Sciences University (Augusta, GA). At University Park, the specimens are stored in a -80ºC freezer in Noll first floor hallway. All specimens not exhausted upon analysis are maintained no longer than 5 years after publication.

9.1.3 Access to Data and/or Specimens

Only authorized personnel have access.

9.1.4 Transferring Data and/or Specimens

Some specimens are mailed or transported by lab members or couriers of outside labs for analysis. All specimens are coded and do not contain identifiers. We do not share the code with unauthorized persons. The outside labs are at Quest Diagnostics (Chantilly, VA), Ohio State University (Columbus, OH), and Georgia Health Sciences University (Augusta, GA).

9.2 Subject Privacy

We tell participants that they may decline to answer questions and decline to participate in the study. Only authorized personnel are present during screening and experiments. On occasion (e.g. educational visit, visiting colleague, site visit) participants may give permission for visitors to observe a procedure or experiment.

10.0 Data and Safety Monitoring Plan

NA

- **10.1 Periodic evaluation of data**
- **10.2 Data that are reviewed**
- **10.3 Method of collection of safety information**
- **10.4 Frequency of data collection**
- **10.5 Individuals reviewing the data**
- **10.6 Frequency of review of cumulative data**
- **10.7 Statistical tests**
- **10.8 Suspension of research**

11.0 Risks

General note: The research group's members are trained and competent in their duties. The group, led by Dr. Alexander, evaluates the effectiveness and safety of protocols and procedures in an ongoing fashion. We discuss the protocol with candidates, invite questions, and offer tours of the laboratory. Prior to medical screening, candidates read

and sign informed consent forms detailing protocols, procedures, risks, sensations, compensation, etc. We give candidates witnessed copies of the signed consent forms. After accepting participants into the study, we discuss and review the procedures and protocols with them generally and at each step throughout the project. We frequently remind participants of the option to withdraw from the study at any time. Restricting access to experiments, data, and coding to authorized personnel maintains confidentiality. The Noll Lab's electronics technician certifies the electrical devices for human use. Lists of emergency numbers remain by lab telephones. At least one cell phone is present at each experiment. A hospital and emergency medical services are within 1-2 miles of the lab. An AED is located nearby in the hallway.

Microdialysis: We insert a 25 g needle horizontally into and then out of the layers of the skin of the ventral forearm. The needle's entry and exit are about 2.5 cm apart. We thread the microdialysis "probe," comprising a tube of membrane (320 um OD) with tubing attached at both ends (650 um OD), through the needle. Then we withdraw the needle leaving the membrane under the skin. We perfuse the probe with sterile saline using a syringe pump.

Cutaneous microdialysis commonly causes some pain and bruising similar to that experienced during blood draws. There is usually no pain after the probe is in place. The participant may experience mild pain while we remove probe. Minor bleeding may occur. As with routine venipuncture, a participant who is nervous about needles could have increased heart rate and blood pressure, become lightheaded or nauseated, or could faint.

As with venipuncture or any event that breaks the skin, infection is possible, but proper aseptic technique and sterile solutions / supplies keep the risk minimal. However, no participants in any of our experiments been reported infection. We place a sterile bandage on the site after the experiment.

The persons performing MD have been trained in proper technique and procedure by Dr. Alexander and had their technique witnessed / approved by Robert Mooney, D.O. , and/or Sarah Ferguson, M.D. (see uploads under respective "CV"s). Ice numbs the skin and the small needle reduces pain during insertion. We stop any bleeding by applying mild pressure to the site with sterile gauze. In the unlikely event that the individual has an allergic reaction, we stop microdialysis immediately. If the reaction becomes severe, we seek emergency medical assistance. Although rare, if the membrane should break in half during removal, we remove the remaining half by gently pulling the attached tubing. This presents no additional risk to the participant. In the unlikely event in which the membrane breaks during removal leaving an isolated piece of membrane under the skin, we treat the piece of membrane in a manner similar to that for a splinter in the skin. In this case, we may have to make a superficial incision for removal. Such an event has not occurred in projects that have used MD in this lab (over 1,000 MD probes have been placed by Dr. Alexander, alone).

Perfusate: Lactated Ringer's solution flows through the microdialysis probes. An allergic reaction to this physiological saline solution is highly unlikely.

All substances (ACh, Apocynin, Ascorbate, L-NAME, SNP, Tempol) added to the lactated Ringer's perfusing the microdialysis probes have been used previously clinically and/or in research in humans.

Microdialysis delivers small amounts of the substances to a nickel-sized area of the skin. The small quantities used and the extremely localized administration during microdialysis does not produce systemic effects. To our knowledge, there are no reports of long or short-term side effects of these substances administered through microdialysis. The chance of adverse reactions to these substances is extremely small given the minute amount delivered to the a very small area of skin, the lack of adverse reactions to similar amounts delivered via MD in many other studies, and lack of adverse effects in human cell cultures. There is a slight chance of allergic reaction to these substances that could produce redness, itching, rash, and/or swelling. A severe reaction (anaphylactic shock) could also cause fever, difficulty in breathing, changes in pulse, convulsions, and/or loss of consciousness.

The perfusate is sterile. We prepare the perfusate in their laboratory using sterile techniques and supplies commonly used for this purpose in research laboratories. The Ringer's solution is sterile as purchased. We add other solutions aseptically to the sterile bag of Ringer's solution through 0.2 micron Gelman Sterile Acrodisc syringe filters. We mix the

perfusate for same-day use and discard excess perfusate after the experiment. During the IND application process, the FDA accepted the researchers' technique for preparing perfusates. Although unlikely, in the case of a severe reaction to the perfusate, the researchers call 911.

Laser Doppler Flowmetry: The probe attaches to the skin with double-sided tape and measures skin blood flow in a 1mm3 volume of skin. Weak lasers can hurt the eye if one should stare into the light for a long time. The red light seen on the surface of the skin is harmless. We do not turn on the laser until they tape the probes to a surface. We remove the tape afterward carefully. We have used this technique in their lab with IRB approval for many years without incident.

Blood pressure (manual and Cardiocap): The manual and Cardiocap methods use a cuff that inflates on the upper arm. The cuff slowly deflates while we listen to the pulse-sounds at the inside of the elbow with a stethoscope or the Cardiocap monitors the pulse through the cuff. The inflated cuff may make the arm feel tingly and numb, and the cuff may temporarily bruise the arm. Efficient and competent measurement technique minimizes the duration of cuff inflation. These techniques to measure blood pressure are unlikely to produce lasting ill effects.

Povidone Iodine: Hospitals and researchers use povidone iodine to clean and sterilize the skin. Participants could be allergic to iodine. An allergic reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse and/or blood pressure, convulsions, shock, and/or loss of consciousness. We use only alcohol on participants with iodine allergy as identified during screening.

Blood draw: Blood draws can cause anxiety (with increased heart rate and blood pressure), mild pain, swelling, nausea, lightheadedness, fainting, or bleeding. There is a slight chance of infection. A competent nurse performs blood draws using standard venipuncture-procedure and techniques that minimize the chance of infection. Participants may recline for the procedure.

Tape and adhesive disks: Participants could be sensitive to the adhesive of the tape and double-sided adhesive disks used in the study causing redness, rash, tenderness, and/or itching. We remove the tape and adhesive disks carefully. Ointment is available, if needed.

Screening: The screening includes blood sample, height, waist circumference, weight, heart rate, blood pressure, pregnancy test, and history performed by the competent research staff. Participants may be uncomfortable giving medical information or being measured. The participants may decline to answer questions or participate in measurements. We conduct screenings professionally and privately.

Initial Screening Form: We use the form to initially determine a candidate's suitability for the study. The initial interview gathers minimally invasive personal data that is kept confidential. We used similar interviews in the past without problem. Candidates may complete the form at the lab group's web site via the Qualtrics commercial survey website. The participant may decline to answer questions. We conduct phone interviews professionally and privately. Only authorized personnel may access completed forms.

ECG: We attach three to twelve electrodes to the participant's chest and then attach the electrode wires to a ECG machine. The machine records the electrical activity of the heart. There are no adverse effects from this measure. A participant may be shy about electrodes applied to the chest. We carefully remove the tape afterward. We conduct the test professionally and privately.

Thermoregulation Lab Website: Potential participants may enter data into the screening form (see above) via the Qualtrics website. Qualtrics is a secure website and survey application designed to support data capture for research studies. The participant may be concerned about data's security. All web traffic to and from the Qualtrics application website is done via a Secure Socket Layer (SSL) that encrypts the data in transmission. The questionnaire contains statements advising of the limitations of technology and that there is no confidentiality guarantee. Data is deleted from

the Qualtric's servers within 24 hours after the user deletes it from the website. Participants may choose a personal interview instead.

Local Heating: The local heating control unit (Moor Instruments) precisely controls and monitors the temperature of the heated probe holders used with the Laser Doppler Flowmeter. To determine the maximal SkBF, we increase the temperature of the heating units slowly (about 0.1°C every 1 second). The skin feels very warm but not painful. Local heating causes temporary redness of the skin that subsides within several hours. This technique is very unlikely to produce long-term ill effects. The local heating controllers (Moor Instruments) precisely control and monitor the temperature of the heated probe holders. The system has programmed maximum temperature limits. This technique is unlikely to cause long-term ill effects.

Latex: Some gloves and medical materials are made of latex rubber. Some people may be sensitive to latex. Latex: Screening identifies and excludes candidates having a known latex allergy.

Controlled Feeding Protocol: It is possible that the subject could have a food allergy. Food allergies can produce redness, itching, rash, and/or swelling. A severe reaction (anaphylactic shock) could also cause fever, difficulty in breathing, changes in pulse, convulsions, and/or loss of consciousness. To avoid known food allergies, screening for this study includes identification of known allergies, including food allergies. Also, subjects meet with a registered dietitian prior to participating to identify food preferences. If a subject is allergic to the investigative substances, she/he is excluded from the study. If a subject is allergic to a substance that may be appropriately substituted with another, we make the substitution, otherwise we exclude the subject from the study.

Flow Mediated Vasodilation (FMD) Test / Doppler Ultrasound: There is a small chance the probe could irritate the skin. Placing the probe on the arm's skin may cause temporary minor redness. The inflated cuffs may cause the participant's arms and feet to feel numb or tingly, and the skin's color to change slightly. The cuffs could cause mild bruising. The gel is the same as that used with medical ultrasound tests. The gel may feel cool or cold on the skin. A bad reaction to the gel is highly unlikely. The cuffs inflate for a minimal amount of time. The temporary redness from the probe is unlikely to have lasting ill effects. The participant may decline the test.

Sublingual Nitroglycerine: Subjects may experience some of the following reactions to the nitroglycerine

headache	lightheadedness	dry mouth	flushing
irregular heart beat	weakness	nausea	vomiting
5-10 minute drop in blood pressure	fainting	dizziness	sweating

Subjects may notice a sweet taste and/or tingling sensation in the mouth while the tablet dissolves. All these effects are usually short-lived. We minimized the effects by having subjects remain supine for 20 minutes after receiving the tablet. In subjects who experience a drop in blood pressure, values usually return to within 10 mmHg of baseline levels upon resolution of the testing. We monitor subjects for up to an hour after they receive the nitroglycerin if they have a strong or bad reaction. Subjects could have a mild or severe allergic response to the drug. This response could include rash, itching, difficulty breathing, and swelling of the face, lips, tongue, or throat. In the event that blood pressure does not return to baseline, coupled with related symptoms, we refer subjects for medical follow-up. In the event of a severe reaction (e.g. anaphylaxis) we call 911.

The effects of nitroglycerin on pregnant or nursing women are unknown. We exclude subjects who are pregnant or nursing. The use of nitroglycerin for artery measurements is not an FDA-approved use of this drug. However, nitroglycerin has been used in this way in many research studies nation-wide without problem. Also, sublingual nitroglycerin is often prescribed for and self-administered by heart patients who have, or are at risk for, angina (heart pain).

Fasting: Subjects may feel hungry during the fasting period and for the duration of experiment. We have conducted many research protocols over the years in which subjects were required to fast 8 hours prior to and then during experiments. In the vast experience of the medical research staff and investigators, there is no risk of fainting due to fasting for this length of time.

12.0 Potential Benefits to Subjects and Others

12.1 Potential Benefits to Subjects

Subjects receive a medical screening that could inform them about their health. They learn their blood pressure and blood cholesterol levels. This is important knowledge. High blood pressure and blood cholesterol contribute to many serious health problems.

12.2 Potential Benefits to Others

One in three adults under the age of 65 and one out of two adults over the age of 60 has high blood pressure. 40% of all deaths in the United States are due to CVD. This health care burden is greater than $350 billion/year and growing. As the population ages, these problems will increase. The study will explore dairy as a candidate for early intervention through a change in diet. This could reduce the need for drug treatment and its attending side effects and cost. The projects provide valuable experience, education and partial fulfillment of degree-work for graduate and undergraduate students of The Pennsylvania State University.

13.0 Sharing Results with Subjects

The researchers give participants copies of their individual lab results. When the researchers complete data analysis, participants may attend an optional presentation of the study's general findings at Noll Lab.

14.0 Subject Stipend (Compensation) and/or Travel Reimbursements

Experiments (1 baseline + 4 controlled feeding periods = 5 of each type of experiment)

FMD/Sublingual Nitroglycerin:	$250.00	$50.00 each
MD local heating:	$425.00	$85 each ($15.00 for each MD probe + $25 for completing MD experiment)
MD ACh dose response:	$575.00	$115.00 each ($15.00 for each MD probe + $40 for completing MD experiment)

Bonus for completing all experiments: $200.00
Total = $1,450.00

For incomplete experiments, the researchers pay an amount of money equal to the part completed. For instance, if a subject completes half of an ACh dose response MD experiment, the subject receives $15.00 for each MD probe inserted + $20 ($20.00 is one-half of $40.00). The researchers may ask subjects to repeat a trial. If subjects agree to repeat a trial, they receive payment for the repeated trial as stated above. They are reimbursed for gasoline if they live more than 20 miles from Noll Lab.

15.0 Economic Burden to Subjects

15.1 Costs

None

15.2 Compensation for research-related injury

It is the policy of the institution to provide neither financial compensation nor free medical treatment for research-related injury. In the event of injury resulting from this research, medical treatment is available but will be provided at the usual charge. Such charges may be paid by the study sponsor as outlined in the research agreement and explained in the consent form.

16.0 Resources Available

16.1 Facilities and locations

The Noll laboratory is a 4-floor (35,000 sq. ft.) free-standing building devoted to basic, clinical, and applied physiological research. Biochemistry laboratories are available for sample processing and analyses. Available equipment includes centrifuges, osmometer, assay plate reader, shaker, washer, NA/K analyzer, spectrophotometer, i-Stat analyzer, refractometer, hemocue, hematocrit system, fraction collector, hydrogen sulphide analyzer, sonicator, microbalances, laser doppler flowmeters, full-field laser perfusion imager, mini-syringe pumps/ controllers, sweat rate monitoring

systems, hospital beds, medical infusion chair, ECG analysis system, finipres, plethysmograph, refrigerators, pharmaceutical refrigerator, -20 C and -80 C freezers, heated/refrigerated circulators, water-perfused suits, local skin cooling/ heating systems, critical care monitors, blood pressure monitors, metabolic cart, gas sterilization equipment, and autoclave facilities. In addition to ample office and laboratory laptop or desktop computers for research and student use, Noll Laboratory has two available servers and a trunk line connection to the University's mainframe computer system. Internal, office and laboratory computers are linked. The laboratory has four PC-based data acquisition systems equipped with Dataq hardware driven by Windaq software, two of which are located in computer controlled environmental chambers equipped with treadmills and bikes. A Clinical Research Center is contiguous to the Noll Laboratory in the 14,000 sq. ft. Elmore wing, and available for the proposed studies. Up to 12 subject beds for overnight (or longer) stays, a metabolic kitchen, medical examination rooms, and several specialized procedure rooms are available. Because the CRC is contiguous with existing laboratory space, it is readily available for research support, e.g., pre-test subject screening. Noll Laboratory has exceptional in-house support facilities including machine and electronics shops.

16.2 Feasibility of recruiting the required number of subjects

We are using recruiting methods that will reach a large percent of the general population. We have employed these methods successfully for earlier projects that targeted the same groups.

16.3 PI Time devoted to conducting the research

The researchers have been conducting similar experiments for many years and are familiar with the duration of the procedures/protocols/projects and the amount of staffing required to complete the project while maintaining their other obligations.

16.4 Availability of medical or psychological resources

The CRC is located within the same building as the Noll Lab. A hospital and emergency medical services are within 1-2 miles of the lab. An AED hangs near the labs in the hallway. A CPR mask hangs in each lab. The faculty, staff, grad students, and post docs in the lab group have current BLS training.

16.5 Process for informing Study Team

The PI and senior lab members train new members in the necessary procedures. The researchers conduct weekly lab meetings. They review any applicable changes or issues.

17.0 Other Approvals

17.1 Other Approvals from External Entities

We submit an IND application to the FDA.

17.2 Internal PSU Committee Approvals

Check all that apply:

☐ Anatomic Pathology – Hershey only – Research involves the collection of tissues or use of pathologic specimens. Upload a copy of the Use of Human Tissue For Research Form on the "Supporting Documents" page in CATS IRB. This form is available on the IRB website at: http://www.pennstatehershey.org/web/irb/home/resources/forms

☐ Animal Care and Use – All campuses – Human research involves animals and humans or the use of human tissues in animals

X Biosafety – All campuses – Research involves biohazardous materials (human biological specimens in a PSU research lab, biological toxins, carcinogens, infectious agents, recombinant viruses or DNA or gene therapy).

☐ Conflict of Interest Review – All campuses – Research has one or more of study team members indicated as having a financial interest.

☐ Radiation Safety – Hershey only – Research involves research-related radiation procedures. All research involving radiation procedures (standard of care and/or research-related) must upload the Radiation Review Form on the "Supporting Documents" page in CATS IRB. This form is available on the IRB website at: http://www.pennstatehershey.org/web/irb/home/resources/forms

X IND/IDE Audit – All campuses – Research in which the PSU researcher holds the IND or IDE or intends to hold the IND or IDE.

☐ Scientific Review – Hershey only – All investigator-written research studies requiring review by the convened IRB must provide documentation of scientific review with the IRB submission. The scientific review requirement may be fulfilled by one of the following: (1) external peer-review process; (2) department/institute scientific review committee; or (3) scientific review by the Clinical Research Center Advisory committee. NOTE: Review by the Penn State Hershey Cancer Institute Scientific Review Committee is required if the study involves cancer prevention studies or cancer patients, records and/or tissues. For more information about this requirement see the IRB website at: http://www.pennstatehershey.org/web/irb/home/resources/investigator

18.0 Multi-Site Research

NA

18.1 Communication Plans
18.2 Data Submission and Security Plan
18.3 Subject Enrollment
18.4 Reporting of Adverse Events and New Information
18.5 Audit and Monitoring Plans

19.0 Adverse Event Reporting

19.1 Adverse Event Definitions

For drug studies, incorporate the following definitions into the below responses, as written:	
Adverse event	Any untoward medical occurrence associated with the use of the drug in humans, whether or not considered drug related
Adverse reaction	Any adverse event caused by a drug
Suspected adverse reaction	Any adverse event for which there is a reasonable possibility that the drug caused the adverse event. Suspected adverse reaction implies a lesser degree of certainty about causality than "adverse reaction". • *Reasonable possibility*. For the purpose of IND safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the drug and the adverse event.
Serious adverse event or Serious suspected adverse reaction	Serious adverse event or Serious suspected adverse reaction: An adverse event or suspected adverse reaction that in the view of either the investigator or sponsor, it results in any of the following outcomes: Death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.
Life-threatening adverse event or	An adverse event or suspected adverse reaction is considered "life-threatening" if, in the view of either the Investigator (i.e., the study site

life-threatening suspected adverse reaction	principal investigator) or Sponsor, its occurrence places the patient or research subject at immediate risk of death. It does not include an adverse event or suspected adverse reaction that had it occurred in a more severe form, might have caused death.
Unexpected adverse event or Unexpected suspected adverse reaction.	An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator brochure, general investigational plan, clinical protocol, or elsewhere in the current IND application; or is not listed at the specificity or severity that has been previously observed and/or specified.

For device studies, incorporate the following definitions into the below responses, as written:	
Unanticipated adverse device effect	Any serious adverse effect on health or safety or any life-threatening problem or death caused by, or associated with, a device, if that effect, problem, or death was not previously identified in nature, severity, or degree of incidence in the investigational plan or IDE application (including a supplementary plan or application), or any other unanticipated serious problem associated with a device that relates to the rights, safety, or welfare of subjects.

19.2 Recording of Adverse Events

In the case of untoward events, those lab members present during the event are included in a debriefing and notes of the meeting are taken. The participant involved in an untoward event is interviewed in person or via telephone concerning the event and the responses are included into the notes for that event.

All adverse events (serious or non-serious) and abnormal test findings observed or reported to study team believed to be associated with the study drug(s) or device(s) will be followed until the event (or its sequelae) or the abnormal test finding resolves or stabilizes at a level acceptable to the investigator.

An abnormal test finding will be classified as an *adverse event* if one or more of the following criteria are met:

- The test finding is accompanied by clinical symptoms
- The test finding necessitates additional diagnostic evaluation(s) or medical/surgical intervention; including significant additional concomitant drug treatment or other therapy

Note: Simply repeating a test finding, in the absence of any of the other listed criteria, does not constitute an adverse event.

- The test finding leads to a change in study drug dosing or discontinuation of subject participation in the clinical research study
- The test finding is considered an adverse event by the investigator.

19.3 Causality and Severity Assessments

The investigator will promptly review documented adverse events and abnormal test findings to determine 1) if the abnormal test finding should be classified as an adverse event; 2) if there is a reasonable possibility that the adverse event was caused by the study drug(s) or device(s); and 3) if the adverse event meets the criteria for a serious adverse event.

If the investigator's final determination of causality is "unknown and of questionable relationship to the study drug(s) or device(s)", the adverse event will be classified as associated with the use of the study drug(s) or device(s) for reporting purposes. If the investigator's final determination of causality is "unknown but not related to the study drug(s) or device(s)", this determination and the rationale for the determination will be documented in the respective subject's case history.

19.4 Reporting of Adverse Reactions and Unanticipated Problems to the FDA

19.4.1 Written IND/IDE Safety Reports

The Sponsor-Investigator will submit a written IND Safety Report (i.e., completed FDA Form 3500A) to the responsible new drug review division of the FDA for any observed or volunteered adverse event that is determined to be a serious and unexpected, suspected adverse reaction. Each IND Safety Report will be prominently labeled, "IND Safety Report", and a copy will be provided to all participating investigators (if applicable) and sub-investigators.

Written IND Safety Reports will be submitted to the FDA as soon as possible and, in no event, later than 15 calendar days following the Sponsor-Investigator's receipt of the respective adverse event information and determination that it meets the respective criteria for reporting.

For each written IND Safety Report, the Sponsor-Investigator will identify all previously submitted IND Safety Reports that addressed a similar suspected adverse reaction experience and will provide an analysis of the significance of newly reported, suspected adverse reaction in light of the previous, similar report(s) or any other relevant information.

Relevant follow-up information to an IND Safety Report will be submitted to the applicable review division of the FDA as soon as the information is available and will be identified as such (i.e., "Follow-up IND Safety Report").

If the results of the Sponsor-Investigator's follow-up investigation show that an adverse event that was initially determined to not require a written IND Safety Report does, in fact, meet the requirements for reporting; the Sponsor-Investigator will submit a written IND Safety Report as soon as possible, but in no event later than 15 calendar days, after the determination was made.

19.4.2 Telephoned IND Safety Reports – Fatal or Life-threatening Suspected Adverse Reactions

In addition to the subsequent submission of a written IND Safety Report (i.e., completed FDA Form 3500A), the Sponsor-Investigator will notify the responsible review division of the FDA by telephone or facsimile transmission of any unexpected, fatal or life-threatening suspected adverse reaction.

The telephone or facsimile transmission of applicable IND Safety Reports will be made as soon as possible but in no event later than 7 calendar days after the Sponsor-Investigator's receipt of the respective adverse event information and determination that it meets the respective criteria for reporting.

19.5 Reporting Adverse Reactions and Unanticipated Problems to the Responsible IRB

Problems Not Requiring Prompt Reporting: The investigators log internal events/problems that are not problems that require prompt reporting to the IRB (i.e., expected and related events) in an accumulative tracking log that is reported to the IRB with the yearly Continuing Progress Report (CPR) and at the close of the study.

Problems Requiring Prompt Reporting: Internal problems that require prompt reporting and are fatal or life-threatening are reported to the IRB in a Problem Report within one weekday of the principal investigator becoming aware of the problem. All other internal problems that require prompt reporting are reported within five weekdays of the principal investigator becoming aware of the event or problem. External problems that require prompt reporting are reported within 30 days of their receipt by the principal investigator. The investigators issue problem reports to the IRB in the case of adverse events that are: (1) unexpected and (2) related/likely related to the research as determined by the Penn State University (PSU). These events include: specific protocol-defined events that require prompt reporting to the sponsor, breach of confidentiality, incarceration of a participant in a protocol not approved to enroll prisoners, an accidental or unintentional deviation to the IRB-approved protocol that involved risks, an emergency protocol deviation take without prior IRB review to eliminate an apparent immediate hazard to a research participant, a complaint of a participant that indicates an unanticipated risk or any complaint that cannot be resolved by the research staff, information that indicates a change to the risks or potential benefits of the research, change in FDA labeling or withdrawal from marketing of the study drug, device or biologic used in this research protocol, or sponsor-imposed suspension for risk. For each problem, the investigator adds the event to an accumulative problem report log included with the Problem Report submitted to the IRB. The investigator submits the log to the IRB with the yearly Continuing Progress Report (CPR) and at the close of the study.

In accordance with applicable policies of The Pennsylvania State University Institutional Review Board (IRB), the investigator will report, to the IRB, any observed or reported harm (adverse event) experienced by a subject or other individual, which in the opinion of the investigator is determined to be (1) unexpected; and (2) probably related to the research procedures. Harms (adverse events) will be submitted to the IRB in accordance with the IRB policies and procedures.

19.6 Unblinding Procedures

NA

19.7 Stopping Rules

Although such events are extremely unlikely to occur, we are prepared to immediately stop experiments and seek medical assistance if the subjects should experience the more serious reactions described in the informed consents, and IRB applications such as signs and symptoms of an allergic reaction, anaphylactic shock, chest pain, dizziness, and fainting. As always, we remind our subjects throughout the protocol that they may stop the experiment at any time. Also, we exercise the discretion to end a subject's participation if the subject should engage in behavior that could jeopardize his/her own health and well-being or that of others. We end the experiments if the subject's systolic or diastolic blood pressures exceed 180 or 110 mmHg, respectively, heart rate exceeds 85% of the age-predicted maximum, or the body's core temperature reaches 39°C (102 °F) or decreases 1°C (1.8 °F) from baseline. Human subjects undergoing the Controlled Feeding cease participation in the study if we determine that the procedure produces undesirable reactions (i.e. sustained blood pressure increase > 140/90 mmHg).

20.0 Study Monitoring, Auditing and Inspecting

20.1 Study Monitoring Plan

20.1.1 Quality Assurance and Quality Control

Dr. Alexander is responsible for the conduct of the study being in compliance with this protocol, with institutional and IRB policies, with Good Clinical Practice guidelines and any other applicable regulatory requirements. She is assisted by members of her lab group. The monitoring by Dr. Alexander is ongoing.

20.1.2 Safety Monitoring

The Principal Investigator will confirm that all adverse events (AE) are correctly entered into the AE case report forms by the coordinator; be available to answer any questions that the coordinators may have concerning AEs; and will notify the IRB, FDA, sponsor and/or DSMB of all applicable AEs as appropriate. All assessments of AEs will be made by a licensed medical professional who is an investigator on the research.

The research coordinator will complete the appropriate report form and logs; assist the PI to prepare reports and notify the IRB, FDA, and/or DSMB of all Unanticipated Problems/SAE's.

The Monitor will confirm that the AEs are correctly entered into the case report forms. The Monitor will confirm that the adverse events are consistent with the source documents and are reported to the appropriate regulatory bodies, as required.

21.0 Future Undetermined Research: Data and Specimen Banking

NA

21.1 Data and/or specimens being stored

21.2 Location of storage

21.3 Duration of storage

21.4 Access to data and/or specimens

21.5 Procedures to release data or specimens

21.6 Process for returning results

22.0 References

Referenced in Section 2

1. Morrison AC, Ness RB. Sodium intake and cardiovascular disease. Annual review of public health. 2011;32:71-90
2. O'Donnell M, Mente A, Yusuf S. Sodium intake and cardiovascular health. Circulation research. 2015;116:1046-1057
3. Astrup A. Yogurt and dairy product consumption to prevent cardiometabolic diseases: Epidemiologic and experimental studies. The American journal of clinical nutrition. 2014;99:1235S-1242S
4. Qin LQ, Xu JY, Han SF, Zhang ZL, Zhao YY, Szeto IM. Dairy consumption and risk of cardiovascular disease: An updated meta-analysis of prospective cohort studies. Asia Pacific journal of clinical nutrition. 2015;24:90-100
5. Yerlikaya O, Acu M, Kinik O. Importance of dairy products in cardiovascular diseases and type 2 diabetes. Critical reviews in food science and nutrition. 2013;53:902-908
6. Jablonski KL, Racine ML, Geolfos CJ, Gates PE, Chonchol M, McQueen MB, Seals DR. Dietary sodium restriction reverses vascular endothelial dysfunction in middle-aged/older adults with moderately elevated systolic blood pressure. Journal of the American College of Cardiology. 2013;61:335-343
7. Greaney JL, DuPont JJ, Lennon-Edwards SL, Sanders PW, Edwards DG, Farquhar WB. Dietary sodium loading impairs microvascular function independent of blood pressure in humans: Role of oxidative stress. The Journal of physiology. 2012;590:5519-5528
8. Crichton GE, Elias MF, Dore GA, Abhayaratna WP, Robbins MA. Relations between dairy food intake and arterial stiffness: Pulse wave velocity and pulse pressure. Hypertension. 2012;59:1044-1051
9. Bodenheimer T. High and rising health care costs. Part 1: Seeking an explanation. Annals of internal medicine. 2005;142:847-854
10. Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, Hong Y. Heart disease and stroke statistics--2007 update: A report from the american heart association statistics committee and stroke statistics subcommittee. Circulation. 2007;115:e69-171
11. Crichton GE, Alkerwi A. Dairy food intake is positively associated with cardiovascular health: Findings from observation of cardiovascular risk factors in luxembourg study. Nutr Res. 2014;34:1036-1044
12. Markey O, Vasilopoulou D, Givens DI, Lovegrove JA. Dairy and cardiovascular health: Friend or foe? Nutrition bulletin / BNF. 2014;39:161-171
13. Agriculture USDo, Services USDoHaH. Dietary guidelines for americans, 2010. 2010
14. Ballard KD, Mah E, Guo Y, Pei R, Volek JS, Bruno RS. Low-fat milk ingestion prevents postprandial hyperglycemia-mediated impairments in vascular endothelial function in obese individuals with metabolic syndrome. The Journal of nutrition. 2013;143:1602-1610
15. Moore LL, Bradlee ML, Singer MR, Qureshi MM, Buendia JR, Daniels SR. Dietary approaches to stop hypertension (dash) eating pattern and risk of elevated blood pressure in adolescent girls. Br J Nutr. 2012;108:1678-1685
16. Livingstone KM, Lovegrove JA, Cockcroft JR, Elwood PC, Pickering JE, Givens DI. Does dairy food intake predict arterial stiffness and blood pressure in men?: Evidence from the caerphilly prospective study. Hypertension. 2013;61:42-47
17. Yoshizawa M, Maeda S, Miyaki A, Misono M, Choi Y, Shimojo N, Ajisaka R, Tanaka H. Additive beneficial effects of lactotripeptides and aerobic exercise on arterial compliance in postmenopausal women. Am J Physiol Heart Circ Physiol. 2009;297:H1899-1903
18. Wang L, Manson JE, Buring JE, Lee IM, Sesso HD. Dietary intake of dairy products, calcium, and vitamin d and the risk of hypertension in middle-aged and older women. Hypertension. 2008;51:1073-1079
19. Ballard KD, Bruno RS. Protective role of dairy and its constituents on vascular function independent of blood pressure-lowering activities. Nutrition reviews. 2015;73:36-50
20. Ballard KD, Kupchak BR, Volk BM, Mah E, Shkreta A, Liptak C, Ptolemy AS, Kellogg MS, Bruno RS, Seip RL, Maresh CM, Kraemer WJ, Volek JS. Acute effects of ingestion of a novel whey-derived extract on vascular endothelial function in overweight, middle-aged men and women. The British journal of nutrition. 2013;109:882-893
21. Ballard KD, Bruno RS, Seip RL, Quann EE, Volk BM, Freidenreich DJ, Kawiecki DM, Kupchak BR, Chung MY, Kraemer WJ, Volek JS. Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: A randomized, placebo controlled trial. Nutrition journal. 2009;8:34
22. Turpeinen AM, Jarvenpaa S, Kautiainen H, Korpela R, Vapaatalo H. Antihypertensive effects of bioactive tripeptides-a random effects meta-analysis. Ann Med. 2013;45:51-56
23. Xu JY, Qin LQ, Wang PY, Li W, Chang C. Effect of milk tripeptides on blood pressure: A meta-analysis of randomized controlled trials. Nutrition. 2008;24:933-940
24. Murakami M, Tonouchi H, Takahashi R, Kitazawa H, Kawai Y, Negishi H, Saito T. Structural analysis of a new anti-hypertensive peptide (beta-lactosin b) isolated from a commercial whey product. Journal of dairy science. 2004;87:1967-1974

25. van der Zander K, Jakel M, Bianco V, Koning MM. Fermented lactotripeptides-containing milk lowers daytime blood pressure in high normal-to-mild hypertensive subjects. J Hum Hypertens. 2008;22:804-806
26. Xia Z, Liu M, Wu Y, Sharma V, Luo T, Ouyang J, McNeill JH. N-acetylcysteine attenuates tnf-alpha-induced human vascular endothelial cell apoptosis and restores enos expression. Eur J Pharmacol. 2006;550:134-142
27. Szabo C, Hardebo JE, Salford LG. Role of endothelium in the responses of human intracranial arteries to a slight reduction of extracellular magnesium. Exp Physiol. 1992;77:209-211
28. Jain SK, Velusamy T, Croad JL, Rains JL, Bull R. L-cysteine supplementation lowers blood glucose, glycated hemoglobin, crp, mcp-1, and oxidative stress and inhibits nf-kappab activation in the livers of zucker diabetic rats. Free Radic Biol Med. 2009;46:1633-1638
29. Tsai GY, Cui JZ, Syed H, Xia Z, Ozerdem U, McNeill JH, Matsubara JA. Effect of n-acetylcysteine on the early expression of inflammatory markers in the retina and plasma of diabetic rats. Clinical & experimental ophthalmology. 2009;37:223-231
30. De Mattia G, Bravi MC, Laurenti O, Cassone-Faldetta M, Proietti A, De Luca O, Armiento A, Ferri C. Reduction of oxidative stress by oral n-acetyl-l-cysteine treatment decreases plasma soluble vascular cell adhesion molecule-1 concentrations in non-obese, non-dyslipidaemic, normotensive, patients with non-insulin-dependent diabetes. Diabetologia. 1998;41:1392-1396
31. Committee DGA. Scientific report of the 2015 dietary guidelines advisory committee 2015
32. Elliott P, Stamler J, Nichols R, Dyer AR, Stamler R, Kesteloot H, Marmot M. Intersalt revisited: Further analyses of 24 hour sodium excretion and blood pressure within and across populations. Intersalt cooperative research group. BMJ. 1996;312:1249-1253
33. Weinberger MH. Salt sensitivity of blood pressure in humans. Hypertension. 1996;27:481-490
34. Aburto NJ, Ziolkovska A, Hooper L, Elliott P, Cappuccio FP, Meerpohl JJ. Effect of lower sodium intake on health: Systematic review and meta-analyses. BMJ. 2013;346:f1326
35. He FJ, Li J, Macgregor GA. Effect of longer term modest salt reduction on blood pressure: Cochrane systematic review and meta-analysis of randomised trials. BMJ. 2013;346:f1325
36. Van Horn L. Dietary sodium and blood pressure: How low should we go? Progress in cardiovascular diseases. 2015
37. Zhu J, Mori T, Huang T, Lombard JH. Effect of high-salt diet on no release and superoxide production in rat aorta. American journal of physiology. Heart and circulatory physiology. 2004;286:H575-583
38. DuPont JJ, Greaney JL, Wenner MM, Lennon-Edwards SL, Sanders PW, Farquhar WB, Edwards DG. High dietary sodium intake impairs endothelium-dependent dilation in healthy salt-resistant humans. Journal of hypertension. 2013;31:530-536
39. Liu Y, Rusch NJ, Lombard JH. Loss of endothelium and receptor-mediated dilation in pial arterioles of rats fed a short-term high salt diet. Hypertension. 1999;33:686-688
40. Lenda DM, Boegehold MA. Effect of a high salt diet on microvascular antioxidant enzymes. Journal of vascular research. 2002;39:41-50
41. Nurkiewicz TR, Wu G, Li P, Boegehold MA. Decreased arteriolar tetrahydrobiopterin is linked to superoxide generation from nitric oxide synthase in mice fed high salt. Microcirculation. 2010;17:147-157
42. Zhu J, Huang T, Lombard JH. Effect of high-salt diet on vascular relaxation and oxidative stress in mesenteric resistance arteries. Journal of vascular research. 2007;44:382-390
43. Jablonski KL, Gates PE, Pierce GL, Seals DR. Low dietary sodium intake is associated with enhanced vascular endothelial function in middle-aged and older adults with elevated systolic blood pressure. Ther Adv Cardiovasc Dis. 2009;3:347-356
44. Tzemos N, Lim PO, Wong S, Struthers AD, MacDonald TM. Adverse cardiovascular effects of acute salt loading in young normotensive individuals. Hypertension. 2008;51:1525-1530
45. Eisenach JH, Gullixson LR, Kost SL, Joyner MJ, Turner ST, Nicholson WT. Sex differences in salt sensitivity to nitric oxide dependent vasodilation in healthy young adults. J Appl Physiol (1985). 2012;112:1049-1053
46. Holowatz LA, Kenney, W. L. Oral atorvastatin therapy increases nitric oxide-dependent cutaneous vasodilation in humans by decreasing ascorbate-sensitive oxidants. Am J Physiol Regul Integr Comp Physiol. 2011;301:R763-768
47. Fujii N, Brunt VE, Minson CT. Tempol improves cutaneous thermal hyperemia through increasing nitric oxide bioavailability in young smokers. American journal of physiology. Heart and circulatory physiology. 2014;306:H1507-1511
48. Medow MS, Bamji N, Clarke D, Ocon AJ, Stewart JM. Reactive oxygen species (ros) from nadph and xanthine oxidase modulate the cutaneous local heating response in healthy humans. J Appl Physiol (1985). 2011;111:20-26
49. Bruning RS, Santhanam L, Stanhewicz AE, Smith CJ, Berkowitz DE, Kenney WL, Holowatz LA. Endothelial nitric oxide synthase mediates cutaneous vasodilation during local heating and is attenuated in middle-aged human skin. Journal of applied physiology: respiratory, environmental and exercise physiology. 2012;112:2019-2026
50. Zhang QX, Jin MM, Zhang L, Yu HX, Sun Z, Lu RR. Hydrophobicity of whey protein hydrolysates enhances the protective effect against oxidative damage on pc 12 cells. The Journal of dairy research. 2015;82:1-7

L-NAME, SNP, ACh

1. Adler, S., H. Huang, K. Loke, X. Xu, A. Laumas, and T. H. Hintze. Modulation of renal oxygen consumption by nitric oxide is impaired after development of congestive heart failure in dogs. J Cardiovasc Pharmacol. 37:301-309, 2001.
2. Adler, S., H. Huang, K. E. Loke, X. Xu, H. Tada, A. Laumas, and T. H. Hintze. Endothelial nitric oxide synthase plays an essential role in regulation of renal oxygen consumption by NO. Am J Physiol Renal Physiol. 280:F838-843, 2001.
3. Binggeli, C., L. E. Spieker, R. Corti, I. Sudano, V. Stojanovic, D. Hayoz, T. F. Luscher, and G. Noll. Statins enhance postischemic hyperemia in the skin circulation of hypercholesterolemic patients: a monitoring test of endothelial dysfunction for clinical practice? J Am Coll Cardiol. 42:71-77, 2003.
4. Buxton, I. L., D. J. Cheek, D. Eckman, D. P. Westfall, K. M. Sanders, and K. D. Keef. NG-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. Circ Res. 72:387-395, 1993.
5. Chauhan, A., R. S. More, P. A. Mullins, G. Taylor, C. Petch, and P. M. Schofield. Aging-associated endothelial dysfunction in humans is reversed by L-arginine. J Am Coll Cardiol. 28:1796-1804, 1996.
6. Cooke, J. P., J. Dzau, and A. Creager. Endothelial dysfunction in hypercholesterolemia is corrected by L-arginine. Basic Res Cardiol. 86 Suppl 2:173-181, 1991.
7. Durante, W., L. Liao, S. V. Reyna, K. J. Peyton, and A. I. Schafer. Transforming growth factor-beta(1) stimulates L-arginine transport and metabolism in vascular smooth muscle cells: role in polyamine and collagen synthesis. Circulation. 103:1121-1127, 2001.
8. Goode, G. K. and A. M. Heagerty. In vitro responses of human peripheral small arteries in hypercholesterolemia and effects of therapy. Circulation. 91:2898-2903, 1995.
9. Holowatz, L. A., C. S. Thompson, and W. L. Kenney. L-Arginine supplementation or arginase inhibition augments reflex cutaneous vasodilatation in aged human skin. J Physiol. 574:573-581, 2006.
10. Holowatz, L. A., C. S. Thompson, C. T. Minson, and W. L. Kenney. Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. J Physiol. 563:965-973, 2005.

Tempol and Ascorbic Acid

1. Adeagbo, A. S., I. G. Joshua, C. Falkner, and P. J. Matheson. Tempol, an antioxidant, restores endothelium-derived hyperpolarizing factor-mediated vasodilation during hypertension. *Eur J Pharmacol*. 481:91-100, 2003.
2. Colleluori, D. M. and D. E. Ash. Classical and slow-binding inhibitors of human type II arginase. *Biochemistry*. 40:9356-9362, 2001.
3. Cross, J. M., A. E. Donald, S. L. Nuttall, J. E. Deanfield, R. G. Woolfson, and R. J. Macallister. Vitamin C improves resistance but not conduit artery endothelial function in patients with chronic renal failure. *Kidney Int*. 63:1433-1442, 2003.
4. Damiani, E., L. Greci, R. Parsons, and J. Knowland. Nitroxide radicals protect DNA from damage when illuminated in vitro in the presence of dibenzoylmethane and a common sunscreen ingredient. *Free Radic Biol Med*. 26:809-816, 1999.
5. Dessolin, J., M. Schuler, A. Quinart, F. De Giorgi, L. Ghosez, and F. Ichas. Selective targeting of synthetic antioxidants to mitochondria: towards a mitochondrial medicine for neurodegenerative diseases? *Eur J Pharmacol*. 447:155-161, 2002.
6. DuPont JJ, Ramick MG, Farquhar WB, Townsend RR, Edwards DG. NADPH oxidase-derived reactive oxygen species contribute to impaired cutaneous microvascular function in chronic kidney disease. *Am J Physiol Renal Physiol*. Jun 15;306(12):F1499-506, 2014.
7. Fujii N, Brunt VE, Minson CT. Tempol improves cutaneous thermal hyperemia through increasing nitric oxide bioavailability in young smokers. *Am J Physiol Heart Circ Physiol.* Jun 1;306(11):H1507-11, 2014.
6. Gwozdzinski, K., G. Bartosz, and W. Leyko. Effect of thiol reagents and ionizing radiation on the permeability of erythrocyte membrane for spin-labeled non-electrolytes. *Radiat Environ Biophys*. 22:53-59, 1983.
7. Hirooka, Y., K. Eshima, S. Setoguchi, T. Kishi, K. Egashira, and A. Takeshita. Vitamin C improves attenuated angiotensin II-induced endothelium-dependent vasodilation in human forearm vessels. *Hypertens Res*. 26:953-959, 2003.
8. Johnstone, P. A., W. G. DeGraff, and J. B. Mitchell. Protection from radiation-induced chromosomal aberrations by the nitroxide Tempol. *Cancer*. 75:2323-2327, 1995.
9. Kimura, S., G. X. Zhang, Y. Nagai, K. Miyata, A. Nishiyama, T. Shokoji, L. Yao, Y. Y. Fan, M. Rahman, Y. Fujisawa, A. Miyatake, and Y. Abe. Time-dependent transition of tempol-sensitive reduction of blood pressure in angiotensin II-induced hypertension. *J Hypertens*. 22:2161-2168, 2004.
10. Klingler, W., L. Kreja, W. Nothdurft, and C. Selig. Influence of different radioprotective compounds on radiotolerance and cell cycle distribution of human progenitor cells of granulocytopoiesis in vitro. *Br J Haematol*. 119:244-254, 2002.

Apocynin

1. DuPont JJ, Ramick MG, Farquhar WB, Townsend RR, Edwards DG. Nadph oxidase-derived reactive oxygen species contribute to impaired cutaneous microvascular function in chronic kidney disease. American journal of physiology. Renal physiology. 2014;306:F1499-1506.
2. Medow MS, Bamji N, Clarke D, Ocon AJ, Stewart JM. Reactive oxygen species (ros) from nadph and xanthine oxidase modulate the cutaneous local heating response in healthy humans. J Appl Physiol (1985). 2011;111:20-26.

Microdialysis

1. Bennett, L. A., J. M. Johnson, D. P. Stephens, A. R. Saad, and D. L. Kellogg, Jr. Evidence for a role for vasoactive intestinal peptide in active vasodilatation in the cutaneous vasculature of humans. *J Physiol*. 552:223-232, 2003.
2. Boutsiouki, P., S. Georgiou, and G. F. Clough. Recovery of Nitric Oxide from Acetylcholine-Mediated Vasodilatation in Human Skin In Vivo. *Microcirculation*. 11:249-259, 2004.
3. Church, M. K., T. J. Griffiths, S. Jeffery, L. C. Ravell, A. S. Cowburn, A. P. Sampson, and G. F. Clough. Are cysteinyl leukotrienes involved in allergic responses in human skin? *Clin Exp Allergy*. 32:1013-1019, 2002.
4. Clough, G. and M. Noble. Microdialysis--a model for studying chronic wounds. *Int J Low Extrem Wounds*. 2:233-239, 2003.
5. Clough, G. F., P. Boutsiouki, M. K. Church, and C. C. Michel. Effects of blood flow on the in vivo recovery of a small diffusible molecule by microdialysis in human skin. *J Pharmacol Exp Ther*. 302:681-686, 2002.
6. Crandall, C. G., M. Shibasaki, and T. C. Yen. Evidence that the human cutaneous venoarteriolar response is not mediated by adrenergic mechanisms. *J Physiol*. 538:599-605, 2002.
7. Holowatz, L. A., B. L. Houghton, B. J. Wong, B. W. Wilkins, A. W. Harding, W. L. Kenney, and C. T. Minson. Nitric oxide and attenuated reflex cutaneous vasodilation in aged skin. *Am J Physiol Heart Circ Physiol*. 284:H1662-1667, 2003.
8. Holowatz, L. A., C. S. Thompson, and W. L. Kenney. Acute ascorbate supplementation alone or combined with arginase inhibition augments reflex cutaneous vasodilation in aged human skin. *Am J Physiol Heart Circ Physiol*. 291:H2965-2970, 2006.
9. Holowatz, L. A., C. S. Thompson, and W. L. Kenney. L-arginine supplementation or arginase inhibition augments reflex cutaneous vasodilatation in aged human skin. *J Physiol*, 2006.
10. Holowatz, L. A., C. S. Thompson, C. T. Minson, and W. L. Kenney. Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. *J Physiol*. 563:965-973, 2005.

Pyrogen References

1. Brown, S., and A.C. Fuller, "Depyrogenation of Pharmaceutical Solutions Using Submicron and Ultrafilters," J. Parenteral Sci. & Tech., Vol. 47, No. 6, 1993, pp. 285-288.

   The effect of varying the pH and ionic strength on endotoxin removal (depyrogenation) from Water for Injections (WFI) was investigated. Studies using submicron filters showed that endotoxin aggregation and filter retention increased with increasing molarity and decreasing pH. Using a Sartorius 0.01 micron filter, greater than 98% endotoxin retention could be achieved with 10 endotoxin units (EU)/ml bulk solution, and greater than 97% endotoxin retention with the 500 EU/ml bulk solution. Depyrogenation of active and placebo solutions of the radiopaque, Iohexol (350 mgI/ml), using ultrafilters of varying nominal molecular weight limit (NMWL 10,000-300,000) and a Pall Posidyne 0.2 micron filter was also investigated. Results with the ultrafilters showed that it was possible to increase the molecular weight cut-off of an ultrafilter from 10,000 to 100,000, without affecting the efficiency of endotoxin removal, thereby increasing flow rate and reducing filtration time. The Posidyne filter was able to depyrogenate Iohexol active and placebo product. The use of submicron filtration in place of ultrafiltration would provide significant cost benefits in terms of filtration time and equipment costs, and they have been shown to be capable of efficient depyrogenation of these pharmaceutical products.

2. McCormick J, Pragman A, Stolpa J, Leung D, Schlievert P, "Funtional Characterization of Streptococcal Pyrogenic Exotoxin J, a Novel Superantigen" Infection and Immunity, Vol. 69, No. 3: p. 1381-1388, 2001.
3. Orwin P, Leung D, Tripp T, Bohach G, Earhart C, Ohlendorf D, Schlievert P. 2002.
   Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. Biochemistry 41:14033-14040, 2002.

   Staphylococcus aureus is an important human pathogen, causing a variety of diseases. Major virulence factors of this organism include staphylococcal enterotoxins (SEs) that cause food poisoning and toxic shock syndrome. Our study identified a novel enterotoxin-like protein that is a member of the new subfamily (group V) of pyrogenic toxin superantigens (PTSAgs) and examined its biochemical and immunobiological properties. The gene encoding the SE-like protein is directly 5' of another recently identified PTSAg, SEK. The SE-like protein had a molecular weight of 26000 and an experimentally determined isoelectric point between 7.5 and 8.0. We demonstrated that the PTSAg had many of the biological activities associated with SEs, including superantigenicity, pyrogenicity, and ability to enhance endotoxin shock, but lacked both lethality in rabbits when administered in subcutaneous miniosmotic pumps and emetic activity in monkeys. Recombinant protein stimulated human CD4 and CD8 T cells in a T cell receptor variable region, β chain (TCRVβ) specific manner. T cells bearing TCRVβ2, 5.1, and 21.3 were significantly stimulated.

4. Orwin P, Fitzgerald JR, Leung D, Gutierrez J, Bohach G, Schlievert P, "Characterization of *Staphylococcus aureus* Entertoxin L, "Infection and Immunity, Vol. 71, No. 5p.: 2916-2919, 2003.

5. Weber C, Linsberger I, Rafiee-Tehrani M, Falkenhagen D: Permeability and adsorption capacity of dialysis membranes to lipid A. *Int J Artif Organs* 20: 144-152, 1997.

Hemodialysis membranes were tested in vitro for possible penetration by low molecular weight endotoxins containing lipid A. Using lipid A from Escherichia coli as a model substance for this kind of pyrogen, different dialyzers (F4, E3. Acepal 1300, Altraflux, F 40, Polyflux 110, Filtral 12, F 60) were challenged by tangential filtration in aqueous medium. All membranes exhibited impermability to lipid A (as well as to LPS from Pseudomonas aeruginosa), which was proved by additional experiments using culture filtrates of Pseudomonas aeruginosa in bicarbonate dialysis fluid, as well as by employing miniaturized dialyzers with synthetic lipid A as a contaminant. Furthermore, the highest adsorption capacities were found for polysulfone and polyamide membranes.

CONSENT FOR RESEARCH
The Pennsylvania State University

Title of Project: Cheese Consumption and Human Microvascular Function

Principal Investigator: Lacy M. Alexander, Ph.D.
Address: 113 Noll Laboratory
Telephone Number: 814-867-1781

Subject's Printed Name: _____

We are asking you to be in a research study. This form gives you information about the research.

Whether or not you take part is up to you. You can choose not to take part. You can agree to take part and later change your mind. Your decision will not be held against you.

Please ask questions about anything that is unclear to you and take your time to make your choice.

1. Why is this research study being done? High blood pressure (hypertension, HT) can lead to disease in the body's blood vessels (cardiovascular disease or CVD). CVD is the leading cause of death in developed countries. High blood pressure and CVD cause harmful changes to the blood vessels in the kidney, heart, and other organs. These changes also occur in the blood vessels in human skin.

Diet changes can help to lower blood pressure without using drugs. Increased dairy intake improves the results of tests that measure the health of blood vessels. People who had mild HT saw a modest drop in blood pressure when they increased their dairy intake. No one knows how dairy improves the function of blood vessels and lowers blood pressure in humans. We plan to explore the effects of cheese intake on the function of blood vessels. Salt in the diet can cause blood pressure to rise. Cheese contains salt. We explore how cheese intake improves the health of blood vessels despite the salt present in the cheese.

It is much easier to see and study the effects of high blood pressure in the blood vessels of the skin. We use "microdialysis" (MD) in this study. With MD, we perfuse some research drugs into nickel-sized areas of skin on your arm. The drugs remain in the small areas and do not go into the rest of your body. The research drugs are not approved by the FDA to treat disease. However, the FDA has approved our using the drugs in this study. We and others have used these drugs in people in research studies for many years without problem.

We recruit people who have normal to mild high blood pressure for this study. We are asking you to be in this research because you fit our criteria for being a subject.

2. What will happen in this research study?

You participate on the circled days or procedures. Please read the descriptions of the circled days. Then write your initials by the circled days or procedures.

We may ask you to repeat a trial, procedure, or test. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.

Note: This study involves the use of drugs that are not approved by the FDA to treat disease. All of the drugs have been used in humans by us or others. The FDA approved the use of the drugs for this study. We dilute the drugs in Lactated Ringer's, a type of saline fluid like that found throughout your body. The drugs are:

Acetylcholine (ACh) – like a substance made by your body; causes blood vessels to dilate
Apocynin - antioxidant
Ascorbate (Vitamin C) - found in many foods such as citrus fruit; antioxidant
L-NAME – blocks the production of nitric oxide
Sodium nitroprusside (SNP) – supplies nitric oxide; causes blood vessels to dilate
Tempol – antioxidant

_____ initial A. <u>Screening Visit</u>

1. You drink only water and do not eat for 12 hours before the screening.
2. The research nurse and/or Clinical Research Center (CRC) staff perform the screening. The staff measures your height and weight, blood pressure (BP), and heart rate (HR). They measure waist circumference. The staff reviews your medical history. Women of childbearing age have a urine pregnancy test.
3. The staff draws 30 ml (2 Tbsp) of blood from a vein in your arm. We send some of the blood to a lab to see if the proteins, blood cells, electrolytes, etc. are within normal levels. We may test the blood for other substances of interest. The researchers do not perform genetic analyses on the blood nor look for presence of disease (e.g. HIV).
4. If you take a thyroid drug, please tell the nurse your thyroid stimulating hormone (TSH) level. If you do not know your TSH level and/or you have not had it measured within 6 months, the nurse draws a blood sample (3.5 ml; 0.2 Tbsp) to measure TSH.
5. You wear a monitor that measures blood pressure for 24 hours. The monitor has a cuff that goes around your arm. A control unit hangs on a strap around your waist, shoulder, or upper arm.

You meet with our registered dietitian. This meeting helps us to plan the food you eat during the controlled feeding-part of this study. The meeting includes recalling your physical activity on the 7 days before the meeting. Also, you discuss food preferences and issues. (The meeting can occur on a separate day.)

_____ initial B. <u>Baseline Experiments</u> (Separate days, the order of the Local Heating and ACh Dose Response MD experiments is random)

1. You complete a 3-day food record.
2. **Preparation for all experiments**
   a. We give you printed and verbal instructions listing what you need to do before you arrive at the lab. Please follow the instructions with care and arrive prepared. If you have questions, please contact us right away.
   b. Do not consume alcohol, niacin supplements, or fish oils for 48 hours before the experiment.
   c. Do not consume caffeine (ex. coffee, tea, Coca Cola, chocolate) for 12 hours before the experiment.
   d. Do not eat anything for 8 hours before the baseline experiments.

e. Drink only water for 8 hours before the baseline experiments.
f. On the day of the experiment
    i. Do not eat anything the morning of experiment.
    ii. It is important for you to be well hydrated for the experiment so you should drink at least 1 glass (8 ounces) of water before you arrive. (Drink only water the morning of experiment.)
    iii. Refrain from hard exercise, physical labor, and any other task in which you to exert yourself more than a leisurely walk.
    iv. We measure your blood pressure, heart rate, and oral temperature.
    v. Women of childbearing age have a urine pregnancy test if they have not had a test within 2 weeks of the experiment.
    vi. We insert the MD probes: You wash the skin on your forearm. We place a tight band around your arm so we can easily see your veins. For each MD site, we make pairs of pen-marks 2.5 cm (1 inch) apart and away from veins. The MD tubing enters and exits your skin at the marks. We remove the tight band. We clean your arm with an orange-colored fluid and alcohol. We place an ice bag on your arm for 5 minutes to numb your skin. Then we insert a thin needle into your skin at each entry mark. The needle's tip travels between the layers of skin for 2.5 cm (1 inch). It leaves your skin at the matching exit mark. We thread the MD tubing through the needle. We withdraw the needle leaving the tubing in your skin. Any redness of your skin subsides in about 60 – 120 minutes.
    vii. We tape a thin probe and its holder over each site where there is MD tubing in your skin. The thin probe measures skin blood flow (SkBF) with a weak laser light. We control the temperature of the holders. The holders start at 34°C (93°F). During the experiment, we measure blood pressure and heart rate.

3. Baseline Experiments – "Day 1"

a. FMD: FMD measures the health of blood vessels.
    i. We place a blood pressure cuff around your forearm.
    ii. We place gel on your upper arm just above the elbow.
    iii. We place a Doppler ultrasound probe on the gel. The ultrasound makes sound waves to measure the size of blood vessels and the speed of the blood.
    iv. We make a "resting" measurement before we inflate the cuff.
    v. The cuff inflates for 5 minutes to stop blood flow to and from the forearm.
    vi. We deflate the cuff and perform a second reading for 3 minutes.

b. Sublingual nitroglycerin: This test also measures the health of blood vessels. Nitroglycerin causes blood vessels to dilate.
    i. The nurse is present throughout the procedure.
    ii. You lie on a bed or recliner.
    iii. We apply a blood pressure cuff on your upper arm.
    iv. As with FMD, we use an ultrasound probe during the test. We place the probe on an artery near your elbow.
    v. A nurse places a 0.4 mg nitroglycerin tablet under your tongue. Then you close your mouth right away. The tablet dissolves in 15-90 seconds. Do not swallow until the tablet dissolves. The effect lasts for 5-10 minutes.
    vi. You lie still for 20 minutes after you received the nitroglycerin. You remain in the lab at least 20 minutes after you receive the nitroglycerin.

vii. You stay in the lab for up to 60 minutes after you received the nitroglycerin if you have a bad or very strong reaction (e.g. drop in blood pressure that lasts longer than usual). We monitor you during this time.

c. MD Experiment - Local Heating (or ACh Dose Response)
   i. This experiment has 5 MD sites.
   ii. Fluid starts flowing through the tubing in your arm while we wait for the redness to end.
   iii. We collect baseline data for 20 minutes.
   iv. We add the drugs to plain fluid at the MD sites.
      Probe 1. Lactated Ringer's only (control)
      Probe 2. Lactated Ringer's + Ascorbate
      Probe 3. Lactated Ringer's + Tempol
      Probe 4. Lactated Ringer's + Apocyanin

   v. We collect second baseline data for 20 minutes.
   vi. Then, we slowly increase the temperature at the MD sites to 42°C (107.6°F).
   vii. When the skin blood flow becomes stable (about 40 minutes), we add LNAME to the tubing at all MD sites.
   viii. When the skin blood flow becomes stable again (about 40 minutes), we stop the drugs.
   ix. We keep skin's temperature at 42°C (107.6°F) at all MD sites. At the same time, only plain fluid + SNP flows through the tubing at all sites. Heating and adding SNP to the fluid help the blood vessels in your skin to dilate.
   x. After about 30 minutes, the experiment ends. We remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can place a bag of ice on the sites for 10 minutes to reduce any bruising that may occur.
   xi. We measure your blood pressure and heart rate before you leave the lab.

4. Baseline Experiments – "Day 2"

a. MD Experiment - ACh Dose Response (or Local Heating)
   i. This experiment has 5 MD sites.
   ii. Fluid starts flowing through the tubing in your arm while we wait for the redness to end.
   iii. We collect baseline data for about 20 minutes.
   iv. We add the test substances to plain fluid in Probe 3, 4, and 5. After about 30 minutes we add LNAME to Probe 2.
      Probe 1. Lactated Ringer's only (control)
      Probe 2. Lactated Ringer's + LNAME
      Probe 3. Lactated Ringer's + Ascorbate
      Probe 4. Lactated Ringer's + Tempol
      Probe 5. Lactated Ringer's + Apocyanin

   v. When the SkBF is stable, we collect a second baseline data for about 20 minutes.
   vi. We add the first amount of ACh to the plain fluid flowing through each probe.
   vii. As the SkBF becomes stable at each probe (about 5 minutes), we proceed to the next amount of ACh.
   viii. Each probe receives 11 increasing amounts of ACh.

ix. After the last amount of ACh ends, we increase the skin's temperature to 42°C (107.6°F) at all MD sites. At the same time, we add SNP to all MD probes. Heating and adding SNP to the fluid help the blood vessels in your skin to dilate.
x. After about 30 minutes, the experiment ends. We remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can place a bag of ice on the sites for 10 minutes to reduce any bruising that may occur.
xi. We measure your blood pressure and heart rate before you leave the lab.

b. You wait at least 3 days before beginning the Controlled Feeding protocols described below.

_____ **initial C. <u>Controlled Feeding Protocols and Experiments</u>**

(the order of the Local Heating and ACh Dose Response is randomized)

1. Controlled Feeding Protocol

a. Following baseline experiments outlined above, you participate 4 Controlled Feeding Periods. Each period lasts 8 days. After each period, you repeat the experiments described above on Days "8" and "9". You maintain the assigned feeding protocol through the first day of experiments. The feeding protocols occur in random order:

low sodium diet devoid of dairy products	(L-Na)
low sodium diet containing cheese	(L-Na+C)
high sodium diet devoid of dairy products	(H-Na)
high sodium diet containing cheese	(H-Na+C)

b. The special kitchen in the CRC prepares all food and drink for the feeding protocol.
c. During the Controlled Feeding Period, you eat *only* the food and drink provided by us.
d. It is important that you eat all of the food we give to you. You must eat all of the cheese. If you have a consistent problem with eating all of the food, please talk to us about this issue.
e. During the Controlled Feeding Period, refrain from antioxidants (e.g. vitamin C) and drugs containing salts (e.g. antacids).
f. You may consume the food / drink at home.
g. Fill out a daily survey about the food and drink that you consumed during the previous day.
h. Visit the CRC kitchen every 1-2 days to pick up food / drink and drop off containers from the previous day or 2.
i. Return any unconsumed food / drink to the kitchen in the containers provided. We measure the unconsumed portions to find out your nutrient intake.

2. **Preparation for Experiments**

a. We give you printed and verbal instructions telling you to do during the 8-day Controlled Feeding periods and before the experiments.
b. You consume the assigned food / drink for 8 days.
c. <u>Day 7 of each Controlled Feeding period</u>
   i. Collect all urine you produce for 24-hour into a container that we supply.
   ii. You undergo 24-hour blood pressure monitoring.
   iii. You wear a monitor that measures blood pressure for 24 hours. If your blood pressure goes up too much after eating the high sodium food, your participation in the study ends.Refrain from caffeine (ex. coffee, tea, Coca Cola, chocolate) for 12 hours before experiments.
   iv. Do not eat anything for 8 hours before the experiments.

v. Drink only water for 8 hours before the experiments.

d. On the day of the experiment
   i. Do not eat anything the morning of experiment.
   ii. It is important for you to be well hydrated for the experiment so you should drink at least 1 glass (8 ounces) of water before you arrive. (Drink only water the morning of experiment.)
   iii. We measure your blood pressure, heart rate, and oral temperature.
   iv. Women of childbearing age have a urine pregnancy test if they have not had a test within 2 weeks of the experiment.
   v. We insert MD probes in the same manner as that described for the Baseline Experiments.
   vi. We tape a thin probe and its holder over each site where there is MD tubing in your skin. The thin probe measures skin blood flow (SkBF) with a weak laser light. We control the temperature of the holders. The holders start at 34°C (93°F). During the experiment, we measure blood pressure and heart rate.

3. Experiments "Day 8"
   a. The nurse draws a blood sample 30 ml (2 Tbsp) blood draw.
   b. FMD (described above)
   c. MD Experiment - Local Heating (or Acetylcholine Dose Response) as described above.
   d. After Day 8's experiment ends, eat the meals we supply for Day 8. Eat as much of Day 8's meals as you can.

4. Experiments "Day 9"
   a. MD Experiment - Acetylcholine Dose Response (or Local Heating) as described above.
   b. After Day 9's experiment ends, eat your normal diet.

5. You wait at least 3 days before beginning the next Controlled Feeding period.

6. You repeat the 3-day food record during one of the washout periods between feeding protocols.

3. What are the risks and possible discomforts from being in this research study?

Microdialysis: The risks are less than that for a blood draw because microdialysis uses only a small, local area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood. You are likely to have some pain and bruising like that from a blood draw. However, we use ice to numb your arm when we insert the tubing. Also, the small needle reduces pain when we insert the tubing. You are not likely to have pain after the tubing is in place. You may feel a little pain when we remove the tubing from your skin. Needles make some people feel sick to their stomach, lightheaded, or may cause them to faint. Although rare, the tubing could break as we remove it from the skin. Then we remove the tubing still in your skin by pulling on the other end of it. This presents no added risk for you. Even rarer, the tubing could break so that a piece of the tubing is left under your skin. In this case, we treat any tubing still in your skin like a splinter. We stop any mild bleeding with mild pressure and sterile gauze. Infection is possible. We keep the risk of infection very small by using sterile techniques and supplies like those used with blood draws. We apply a sterile bandage to the site after the experiment. We tell you how to take care of the site.

Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm2 (0.4 inch2) area of skin at each tubing site. The amount that enters the skin is very small. However, there is

a chance of having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or fainting. We and other researchers have used these substances with microdialysis in skin. There have been no reports that these substances caused bad reactions. If a bad reaction should occur, we summon medical help.

Lactated Ringer's Solution and normal saline: These fluids are similar to the natural fluids in your skin. The fluids contain salt, potassium, lactate (Ringer's only), and chloride. The acid content is like that your body's natural fluids. A bad reaction to these fluids is highly unlikely.

ACh, Apocynin, ascorbate, LNAME, SNP, and Tempol: These substances stop or mimic the action of your body's natural chemicals upon the blood vessels in the skin. A small amount of these substances enter the skin around the tubing. This only affects the blood flow in the vessels in that nickel-sized area of skin. The effect of these substances is gone within an hour after the experiment.

Laser Doppler Flowmetry: Weak lasers can hurt your eye if you stare into the light for a long time. We do not turn on the laser until the probes are taped to a surface. The tape may irritate your skin.

Blood Pressure (manual, Cardiocap): We measure blood pressure with the method used in a doctor's office. A cuff inflates on the upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in the elbow. Or the Cardiocap monitors blood pressure through the cuff on your upper arm. During the short time cuff inflates, your arm may feel numb or tingly. The cuff could cause mild bruising.

Povidone Iodine: Researchers and hospitals use this orange-colored fluid to clean the skin. You could have a bad reaction to the fluid if you are allergic to iodine. You inform us if you have this allergy. In this case, we use only alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or fainting.

Blood Draw: Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. If you are nervous about needles, blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. We keep the chance of infection minimal with the same techniques used in hospitals. Do not exercise hard for 24 hours before a blood draw.

Tape and sticky disks: The tape or sticky disks could cause a rash. During screening, you tell us if you are sensitive to tape. If a disk sticks very strongly, removing the disk could cause an abrasion like a rug-burn on your skin. An abrasion can feel tender or slightly painful, and can increase risk of infection. If you are sensitive to tape, you may have an increased chance for abrasion. An abrasion has occurred only twice during the years that the disks have been used in similar studies in our lab. We may use an adhesive remover like that used in a doctor's office to remove the disks. If you get an abrasion a nurse checks the site. Antibiotic ointment and a sterile bandage are applied. We tell you how to take care of the site. You could have an allergic reaction to the adhesive remover. The reaction could include rash, itching, fever, or breathing problems. Also, it could include changes in pulse, and/or blood pressure, convulsions, shock, and/or fainting. If a bad reaction should occur, we summon medical help right away.

Medical Screening: You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. You may request someone of the same sex to conduct parts of the screening.

Initial screening form: Only members of our lab group use this form. We use the form to help decide whether you are a good candidate for the study. You may feel shy about answering questions. You may request someone of the same sex to ask you the questions. We collect the information in a private and professional manner. We keep the completed form confidential and secure.

ECG: This machine measures the electrical activity of your heart to record heart rate. You have 3 wires from the machine taped to spots on your chest. There are no adverse effects. The tape may irritate.

Local heating: We measure the temperature of your skin under the holders. During heating, the skin feels very warm but does not hurt. The heating makes the skin under the holder red like when you take a hot bath. The redness goes away within several hours. Some people may be more sensitive to heating. If your arm feels too hot, tell us, and we reduce or stop the heating.

Controlled Feeding Protocol: Food allergies can produce redness, itching, rash, and/or swelling. A severe reaction (anaphylactic shock) could cause fever, problems breathing, and changes in pulse. A severe reaction could include convulsions, and/or loss of consciousness. Please inform the nurse during screening of your known food allergies. Also, inform the dietician when you meet with her. We may be able to replace the problem-food with a food to which you are not allergic without affecting the study. If this is not possible, you cannot be in the study.

FMD Test / Doppler Ultrasound: There is a small chance the probe could irritate the skin. Minor redness may occur where the researchers place the probe against the arm. This is temporary. While the researchers inflate the cuffs, the arms and feet may feel numb or tingly, and the color of the skin may change slightly. The cuffs could cause mild bruising. The gel is the same as that used with medical ultrasound tests. The gel may feel cool or cold on the skin. A bad reaction to the gel is highly unlikely.

Sublingual Nitroglycerine: The research-use of nitroglycerin for artery measurements is not an FDA-approved use of this drug. However, nitroglycerin has been used in this way in many research studies without problem. Nitroglycerin is FDA approved for the treatment of angina (heart pain). The drug is often prescribed for heart patients who have, or are at risk for, angina.

You may have some of the following reactions to the nitroglycerine

headache	lightheadedness	dry mouth	flushing
irregular heart beat	weakness	nausea	vomiting
5-10 minute drop in blood pressure	fainting	dizziness	sweating

You may also notice a sweet taste and/or tingling in your mouth while the tablet dissolves. All these effects are usually short-lived. We can reduce some of them by having you lie down for 20 minutes after you receive the tablet. If your blood pressure drops, it is likely to return to within 10 mmHg of your starting level by the time the test ends. We monitor you for up to an hour after you receive the nitroglycerin if you have a strong or bad reaction. If your blood pressure does not return to baseline, and you have related symptoms (e.g. dizziness) we advise you to see your doctor. You could have a mild or severe allergic response to the drug. This response could include rash, itching, difficulty breathing, and

swelling of your face, lips, tongue, or throat. If you have a severe reaction (e.g. severe allergic response) we call 911.

The effects of nitroglycerin on pregnant or nursing women are unknown. You are not to be in the study if you are pregnant or nursing.

Fasting: You may feel hungry after fasting for the experiments and during the experiments.

There is a risk of loss of confidentiality if your information or your identity is obtained by someone other than the investigators, but precautions will be taken to prevent this from happening. The confidentiality of your electronic data created by you or by the researchers will be maintained to the degree permitted by the technology used. Absolute confidentiality cannot be guaranteed.

4. What are the possible benefits from being in this research study?

4a. What are the possible benefits to you?

You receive a medical screening that could inform you about your health. You learn your blood pressure and blood cholesterol levels. This is important knowledge. High blood pressure and blood cholesterol contribute to many serious health problems. If you have high blood pressure or blood cholesterol, we advise you to work with a health care provider to keep your levels controlled.

4b. What are the possible benefits to others?

One in three adults under the age of 65 and one out of two adults over the age of 60 has high blood pressure. 40% of all deaths in the United States are due to CVD. This health care burden is greater than $350 billion/year and growing. As the population ages, these problems will increase. This study explores dairy as a candidate for early intervention through a change in diet. This could reduce the need for drug treatment and its attending side effects and cost. The projects provide valuable experience, education and partial fulfillment of degree-work for graduate and undergraduate students of The Pennsylvania State University.

5. What other options are available instead of being in this research study?

You may decide not to participate in this research.

6. How long will you take part in this research study?

Screening (1 Visit)	less than 1.5 hour
FMD/MD Experiments (5 Visits)	less than 7 hours each
MD Experiments (5 Visits)	5 hours
Obtain Food, 24-hour BP monitor & urine container, etc. (~14 Visits)	about 15 minutes each

Total: about 75 Hours (about 25 visits; It could take about 8 weeks to complete the study.)

7. How will your privacy and confidentiality be protected if you decide to take part in this research study?

We make efforts to limit the use and sharing of your personal research information to people who have a need to review this information.

- We keep the list that matches your name with your code number in a locked file or password protected file on a computer in a room that is locked when unoccupied. Only authorized members of the lab have access to the list.

- We label your research records with your code number and keep them in a locked file or password protected computer in a room that is locked when unoccupied.

We label your research samples with your code number. We keep the samples in a dedicated ultralow freezer in Noll Lab until analysis.

In the event of any publication or presentation resulting from the research, we do not share your personally identifiable information.

We will do our best to keep your participation in this research study confidential to the extent permitted by law. However, it is possible that other people may find out about your participation in this research study. For example, the following people/groups may check and copy records about this research.

- The Office for Human Research Protections in the U. S. Department of Health and Human Services
- The Food and Drug Administration (FDA)
- The research study sponsor, Dairy Management, Inc.
- The Institutional Review Board (a committee that reviews and approves research studies) and
- The Office for Research Protections.

Some of these records could contain information that personally identifies you. Reasonable efforts will be made to keep the personal information in your research record private. However, absolute confidentiality cannot be guaranteed.

A description of this clinical trial will be available on http://www.clinicalTrials.gov, as required by U.S. Law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

8. What are the costs of taking part in this research study?

8a. What will you have to pay for if you take part in this research study?

Nothing.

8b. What happens if you are injured as a result of taking part in this research study?

In the unlikely event you become injured as a result of your participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

9. Will you be paid or receive credit to take part in this research study?

Experiments (1 baseline + 4 controlled feeding periods = 5 of each type of experiment)

FMD/Sublingual Nitroglycerin:	$250.00	$50.00 each
MD local heating:	$425.00	$85 each ($15.00 for each MD probe + $25 for completing MD experiment)
MD ACh dose response:	$575.00	$115.00 each ($15.00 for each MD probe + $40 for completing MD experiment)
Bonus for completing all experiments:	$200.00	
Total =	$1,450.00	

For incomplete experiments, we pay an amount of money equal to the part completed. For instance, if you complete half of an ACh dose response MD experiment, you receive $15.00 for each MD probe inserted + $20 ($20.00 is one-half of $40.00). We may ask you to repeat a trial. If you agree to repeat a trial, you receive payment for the repeated trial as stated above. We reimburse for gasoline if you live more than 20 miles from Noll Lab.

10. Who is paying for this research study?
Dairy Management, Inc. is paying for this study.

11. What are your rights if you take part in this research study?

- You do not have to be in this research.
- If you choose to be in this research, you have the right to stop at any time.
- If you decide not to be in this research or if you decide to stop at a later date, there will be no penalty or loss of benefits to which you are entitled.
- If you choose to withdraw from the study, all data collected up to the point of withdrawal will remain part of the study and may not be removed.

The person in charge of the research study or the sponsor can remove you from the research study without your approval. We remove you from the study if your blood pressure increases too much during a Controlled Feeding period. Other possible reasons for removal from the study include if we deem that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document. You may decline to answer certain questions. You may decide not to comply with certain procedures. However, your being in the study may be contingent upon answering these questions or complying with the procedures.

During the course of the research you will be provided with any new information that may affect your health, welfare or your decision to continue participating in this research.

12. If you have questions or concerns about this research study, whom should you call?
Please call:

- Study head, Lacy M. Alexander, Ph.D. (W: 814-867-1781)
- The research nurse, Susan Slimak RN (W: 814-863-8556, H: 814-237-4618)
- Dr. Alexander's assistant, Jane Pierzga (W: 814-865-1236, H: 814-692-4720)

if you:

- Have questions, complaints or concerns about the research.
- Believe you may have been harmed by being in the research study.

You may also contact the Office for Research Protections at (814) 865-1775, ORProtections@psu.edu if you:

- Have questions regarding your rights as a person in a research study.
- Have concerns or general questions about the research.
- You may also call this number if you cannot reach the research team or wish to talk to someone else about any concerns related to the research.

INFORMED CONSENT TO TAKE PART IN RESEARCH

Signature of Person Obtaining Informed Consent

Your signature below means that you have explained the research to the subject or subject representative and have answered any questions he/she has about the research.

_____ _____ _____
Signature of person who explained this research Date Printed Name
(Only approved investigators for this research may explain the research and obtain informed consent.)

Signature of Person Giving Informed Consent

Before making the decision about being in this research you should have:

- Discussed this research study with an investigator,
- Read the information in this form, and
- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

Signature of Subject

By signing this consent form, you indicate that you voluntarily choose to be in this research and agree to allow your information to be used and shared as described above.

_____ _____ _____
Signature of Subject Date Printed Name