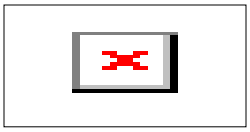
# Nucleophilic Defense Against PM Toxicity (NEAT) Trial

## NCT03314987

## Protocol 5/14/2020



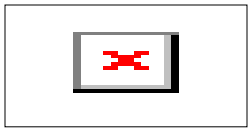
# STUDY PROTOCOL:

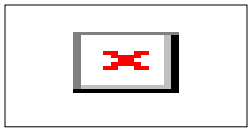
# Nucleophilic Defense against PM Toxicity (NEAT) Trial


## PRINCIPAL INVESTIGATORS:

**Timothy O'Toole, PhD**
**Christina Lee Brown Envirome Institute**
**University of Louisville School of Medicine**

**Aruni Bhatnagar, PhD**
**Christina Lee Brown Envirome Institute**
**University of Louisville School of Medicine**





# 1. PRIMARY INFORMATION

**FULL TITLE**: Nucleophilic Defense against PM Toxicity (NEAT) Trial

**SHORT TITLE**: Carnosine NEAT Trial

**STUDY INSTITUTION(S)**:
Christina Lee Brown Envirome Institute, University of Louisville, Louisville, KY
University of Louisville School of Medicine, Division of Environmental Medicine, Louisville, KY
Clinical Trials Unit, University of Louisville, Louisville, KY

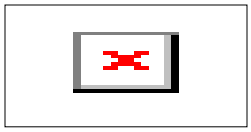
**COLLABORATING INSTITUTION(S)**:
Bellarmine University, Louisville, KY
University of Colorado, Skaggs School of Pharmacy, Aurora, CO

**STUDY SPONSOR**: National Institutes of Health (5R01ES019217-07)

**CLINICALTRIALS.GOV #** NCT03314987

**PRINCIPAL INVESTIGATOR**: Timothy O'Toole, PhD – Christina Lee Brown Envirome Institute, University of Louisville School of Medicine
302 E. Muhammad Ali Blvd, Room 309, Louisville, KY 40202
Office: 502-852-5886
Tim.OToole@Louisville.Edu

**ETHICS BOARD REVIEW**:
University of Louisville Institutional Review Board (IRB)
Approval # 17.1067
Approval Status: Current



# 2. INVESTIGATORS & PERSONNEL

## PRINCIPAL INVESTIGATORS

**Timothy O'Toole, PhD**
Co-Principal Investigator
Christina Lee Brown Envirome Institute, University of Louisville School of Medicine
302 E. Muhammad Ali Blvd, Room 309
Louisville, KY 40202
Office Phone: 502-852-5886
Tim.otoole@Louisville.Edu

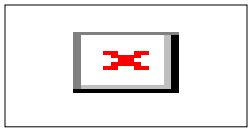
**Aruni Bhatnagar, PhD**
Co-Principal Investigator
Director, Christina Lee Brown Envirome Institute, University of Louisville
302 E. Muhammad Ali Blvd, Room 106
Louisville, KY 40202
Office Phone: 502-852-5873
Aruni@Louisville.Edu

## CO-INVESTIGATORS

**Alok Amraotkar, MD**
Study Management, Primary Contact
Christina Lee Brown Envirome Institute
University of Louisville School of Medicine
302 E. Muhammad Ali Blvd, Room 209
Louisville, KY 40202
Office Phone: 502-852-7559
Alok.Amraotkar@Louisville.Edu

**Andrew DeFilippis, MD**
Study Physician
Division of Cardiovascular Medicine,
University of Louisville School of Medicine
580 S. Preston Street, Room 404-A
Louisville, KY 40202
Office Phone: 502-852-2459
Andrew.DeFilippis@Louisville.Edu

**Rachel Keith, PhD, ARNP**
Data and Safety Monitoring Board Member

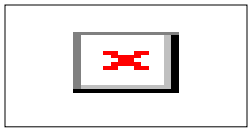


Christina Lee Brown Envirome Institute,
University of Louisville School of Medicine
302 E. Muhammad Ali Blvd, Room 207
Louisville, KY 40202
Office Phone: 502-852-4211
Rachel.Keith@Louisville.Edu

**Shesh N. Rai, PhD**
Study Biostatistician
Department of Biostatistics and Bioinformatics, University of Louisville
Louisville, KY 40202
Office Phone: 502-852-4030
Shesh.Rai@Louisville.Edu

## REGULATORY AND LOGISTICS SUPPORT

**Clinical Trials Unit**
University of Louisville
501 East Broadway, Suite 210
Louisville, KY 40202
Office Phone: 502-852-7559
CTU@Louisville.Edu



# 3. STUDY COMMITTEES

## Executive Committee (EC)

The EC will comprise of three components: study PI, study Medical and Safety Unit, and UofL Clinical Trials Unit. The EC will advise the study PI on various aspects of the study.

1. **Timothy O'Toole, PhD**
   Co-Principal Investigator
   Christina Lee Brown Envirome Institute, University of Louisville School of Medicine
   302 E. Muhammad Ali Blvd, Room 309
   Louisville, KY 40202
   502-852-5886
   Tim.otoole@Louisville.Edu

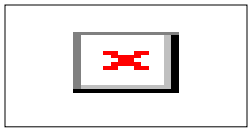
2. **Andrew DeFilippis, MD**
   Co-Investigator, Study Physician
   Division of Cardiovascular Medicine,
   University of Louisville School of Medicine
   580 S. Preston Street, Room 404-A
   Louisville, KY 40202
   Office Phone: 502-852-2459
   Andrew.DeFilippis@Louisville.Edu

3. **Clinical Trials Unit**
   Regulatory and Logistics Support
   University of Louisville
   501 East Broadway, Suite 210
   Louisville, KY 40202
   502-852-7559
   CTU@Louisville.Edu

## Data Safety Monitoring Board (DSMB)

The DSMB will comprise of three independent experts responsible for reviewing clinical trial data on an ongoing basis to ensure the safety of study participants and validity and integrity of the data. The DSMB will use a combination of, notifications, event specific reports, and scheduled cumulative trial reports to keep the Executive Committee (EC) and the Institutional Review Board (IRB) informed about real and potential safety issues.

1. **Matthew Cave, MD**
   Associate Professor of Medicine



Division of Gastroenterology, Hepatology, and Nutrition,
University of Louisville School of Medicine
Louisville, KY 40202
Office Phone: 502-852-6991
Matt.Cave@Louisville.Edu

2. **Shahab Ghafghazi, MD**
Assistant Professor of Medicine,
University of Louisville School of Medicine
Office Phone: 502-852-7959
Shahab.Ghafghazi@Louisville.Edu

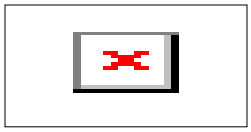
3. **Rachel Keith, PhD, ARNP**
Assistant Professor of Medicine
Christina Lee Brown Envirome Institute,
University of Louisville School of Medicine
302 E. Muhammad Ali Blvd, Room 207
Louisville, KY 40202
Office Phone: 502-852-4211
Rachel.Keith@Louisville.Edu

4. **Shesh N. Rai, PhD**
Professor of Bioinformatics and Biostatistics
Biostatistics and Bioinformatics Facility
Department of Biostatistics and Bioinformatics, University of Louisville
Louisville, KY 40202
502-852-4030
Shesh.Rai@Louisville.Edu

## Randomization Committee (RC)

The RC will be in possession of the randomization key so that accurate unblinding of the randomization assignment may be identified in event of a medical or other emergency. The randomization key will be held by four individuals: the pharmacist compounding the supplement (Dr. Rice), the study physician (Dr. DeFilippis), a member of the UofL Clinical Trials Unit, and the study biostatistician (Dr. Rai).

1. **Peter Rice, PharmD, PhD**
Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado
12850 East Montview Blvd C238
Aurora, CO 80045
303-724-2613

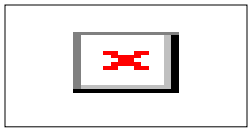


Peter.Rice@CUAnschutz.Edu

2. **Andrew DeFilippis, MD**
   Co-Investigator – Study Physician
   Division of Cardiovascular Medicine,
   University of Louisville School of Medicine
   580 S. Preston Street, Room 404-A
   Louisville, KY 40202
   Office Phone: 502-852-2459
   Andrew.DeFilippis@Louisville.Edu

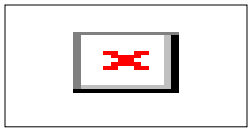
3. **Clinical Trials Unit**
   University of Louisville
   501 East Broadway, Suite 210
   Louisville, KY 40202
   502-852-7559
   CTU@Louisville.Edu

4. **Shesh N. Rai, PhD**
   Professor of Bioinformatics and Biostatistics
   Biostatistics and Bioinformatics Facility
   Department of Biostatistics and Bioinformatics, University of Louisville
   Louisville, KY 40202
   502-852-4030
   Shesh.Rai@Louisville.Edu



# 4. LIST OF ABBREVIATIONS

| | |
|---|---|
| AE | Adverse Event |
| EPC | Endothelial Progenitor Cells |
| CTU | Clinical Trials Unit, University of Louisville |
| DHHS | Department of Health and Human Services |
| DSHEA | Dietary Supplement Health and Education Act of 1994 |
| DSMB | Data Safety Monitoring Board |
| EC | Executive Committee |
| FDA | Food and Drugs Administration |
| IRB | Institutional Review Board |
| MedDRA | Medical Dictionary for Regulatory Activities |
| NEAT | Nucleophilic Defense Against PM Toxicity |
| SAE | Serious Adverse Event |
| SAR | Suspected Adverse Reaction |
| SOC | System Organ Classification |
| SSAR | Serious Suspected Adverse Reaction |
| Supplement | Dietary Supplement Freely Available Over the Counter |
| UofL | University of Louisville |
| UPs | Unanticipated Problems |
| CVD | Cardiovascular Disease |
| WHO | World Health Organization |
| PMA | Platelet Monocyte Aggregates |
| CAP | Concentration Air Particles |
| OC | Organic Carbons |
| $PM_{2.5}$ | Particulate Matter with diameter less than 2.5 micrometers |
| VOC | Volatile Organic Compounds |



# 5. EXECUTIVE SUMMARY

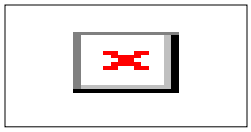
**Study Design**: This is a randomized, double-blind, placebo-controlled longitudinal clinical intervention study to assess the physiologic effects of dietary supplementation of carnosine in healthy human participants. Carnosine is a naturally synthesized peptide in the human body, and is also commercially available as a dietary supplement. The primary endpoint in this study are endothelial progenitor cells (EPCs) found in peripheral blood. Exposure to $PM_{2.5}$ is known to decrease levels of EPCs, which represent angiogenesis.

EPCs will be compared between carnosine and placebo groups at 6 weeks and also their change from baseline to 6 weeks to 12 weeks post initiation of intervention. The secondary endpoints are difference in EPCs at 12 weeks between carnosine versus placebo groups and difference in arterial stiffness, endothelial microparticles, and platelet monocyte aggregates between carnosine versus placebo groups at 6 weeks and 12 weeks.

**Target Population**: Eligible study candidates will be males or females from all ethnicities, between 22 and 65 years of age, living within 30 miles radius of Louisville, KY, willing to provide a urine sample, and willing to participate in the entire study if found eligible for the main study. Eligible candidates will be identified from an existing database of people interested in being contacted for research studies or the University of Louisville outpatient clinics. Study information will be advertised using flyers, social media, and other outreach methods to reach out to a broader population and increase broader representation. Study candidates must agree to the consent and will have no indication of concurrent illness. We will exclude individuals with active treatment for alcohol and drug abuse, or those using immunosuppressant agents or any other medical condition that might compromise the successful completion of the study. Subjects will be prescreened, and those with conditions known to effect peripheral blood cell counts and bone marrow function will be excluded from the study. These conditions included malignancies, organ transplant, renal replacement therapy, type 1 diabetes, untreated thyroid disease, anemia, acute infections, HIV infection, hepatitis, and unhealed wounds. Subjects on hormone replacement therapy or medications affecting bone marrow function or peripheral blood cell counts will also be excluded from the study as will those unwilling or unable to provide informed consent, pregnant or lactating women, prisoners and other vulnerable populations. Personal identifiers will be recorded and secured by clinical personnel.

Eligible candidates will be identified via a screening visit during which the following data will be collected: primary contact and demographic information, and a urine sample. The urine sample will be used to assess levels of carnosine. Individuals with less than median level of carnosine will be invited to participate in the study.

**Selected Sample**: A total of 240 participants will be recruited (120 receiving Carnosine dietary supplementation and 120 receiving placebo); males and females, all ethnicities, who live in

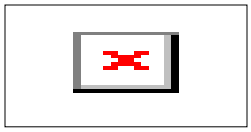


Louisville and surrounding Kentucky counties, with carnosine levels below the median value of the population. The participants should also agree to complete 4 study visits (1 screening, 1 baseline assessment, and 2 follow-ups) and to comply with study intervention regimen. Accounting for approximately 40% attrition, the goal is to randomize a total of 400 participants – 200 in the carnosine group and 200 in the placebo group.

**Enrollment Period**: Study enrollment will be split into two consecutive years, with a goal of equal enrollment each year (60 carnosine supplementation and 60 placebo in 2020; and 60 carnosine supplementation and 60 placebo in 2021). There will be a total of four study visits: Screening (Visit # 1), Baseline Assessment (Visit # 2), Follow-Up # 1 (Visit # 3), and Follow-Up # 2 (Visit # 4). Screening can occur throughout the year. Baseline assessment will occur from May through June. Follow-up # 1 will occur, at 6 weeks post initiation of study supplement, from June through August. Follow-up # 2 will occur, at 12 weeks post initiation of study supplement, from July through September. Study intervention (carnosine supplementation or placebo) will begin in May and continue through September.

**Rationale**: Carnosine is a naturally occurring peptide that is also available commercially as a dietary supplement. Since carnosine has anti-oxidant properties and air pollution exposure induces a state of oxidative stress, the purpose of this study is to see if those taking carnosine as a dietary supplement are protected from air pollution-induced oxidative stress and adverse cardiovascular outcomes.

The protective effects of carnosine against $PM_{2.5}$ toxicity are consistent with the biological role of this peptide and its physiochemical properties. Carnosine is a dipeptide (β-alanyl-L-histidine), which is present in very high concentration in the skeletal muscle, brain and heart. The concentration of carnosine in human skeletal muscle exceeds 10mM, whereas its concentration in brain and heart is between 0.1 to 1mM. High levels of carnosine have also been detected in monocytes and red blood cells. Because of its high buffering capacity as a zwitterion, carnosine is thought to prevent proton-induced inhibition of glycolysis, and sustain glycolytic activity under reduced oxygen concentrations. Hence, carnosine, or its precursor – β-alanine, are widely used as performance enhancing supplements. In addition to maintaining intracellular pH, carnosine has antioxidant, anti-inflammatory and anti-glycating activities. Because of the high nucleophilicity of its histidyl ring, carnosine binds avidly to α,β-unsaturated aldehydes such as acrolein[11] and 4-hydroxy-*trans*-2-nonenal (HNE), which are major products of lipid peroxidation and important “second messengers” of ROS toxicity. Carnosine also reduces the formation of advance glycation end-products (AGEs) that contribute to the progression of several degenerative diseases including diabetes and atherosclerosis. Carnosine supplementation has been shown to prevent inflammation and has beneficial immunomodulatory effects, and our studies have shown that carnosine facilitates the removal of lipid peroxidation products and decreases atherosclerosis in mice as well.



We have identified multiple adverse outcomes of $PM_{2.5}$ exposure in humans including endothelial damage, platelet activation, immune response dysfunction and a depletion of circulating EPCs which are involved in many physiological functions. We have obtained similar results in animal models, which show that exposure to concentrated ambient particles depletes EPCs, and results in defective hematopoiesis. Mechanistic studies from our group and others suggests these diverse outcomes result from the induction of oxidative stress. Hence, carnosine supplementation represents a viable option for attenuating the oxidative and inflammatory effects of $PM_{2.5}$ exposure. Because of its anti-inflammatory, antioxidant, buffering, metal chelating roles,[2 2] carnosine may be more effective than other dietary supplements tested to-date. Moreover, our preliminary data show that carnosine levels are significant determinants of $PM_{2.5}$ toxicity, and, in preclinical models, effective in attenuating the vascular effects of $PM_{2.5}$.

**Primary Endpoints (reflecting change from baseline to 6 weeks and 12 weeks and between groups)**

1. Difference in EPC-2 between Carnosine and Placebo Groups
2. Difference in EPC-3 between Carnosine and Placebo Groups
3. Difference in EPC-4 between Carnosine and Placebo Groups
4. Difference in EPC-7 between Carnosine and Placebo Groups
5. Difference in EPC-8 between Carnosine and Placebo Groups

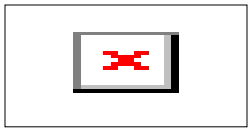
**Secondary Endpoints (reflecting change from baseline to 6 weeks and 12 weeks and between groups)**

1. Difference in Arterial Stiffness between Carnosine and Placebo Groups
2. Difference in Endothelial Microparticles between Carnosine and Placebo Groups
3. Difference in Platelet Monocyte Aggregates between Carnosine and Placebo Groups

**Subgroup Analyses**: First series of analyses will include an examination of the effects of carnosine supplementation on sub-groups:

1. Age
2. Sex
3. Race
4. Socioeconomic Status
5. Diabetes
6. Smoking
7. Hypertension

**Hypothesis (for Primary and Secondary Endpoints)**:
Carnosine supplementation blunts adverse effects of $PM_{2.5}$ pollution on vasculature of healthy participants.



# 6. STUDY OBJECTIVES

## Primary Objective

The primary purpose of this study is to determine whether taking carnosine as a dietary supplement protects the vasculature from $PM_{2.5}$ air pollution-induced oxidative stress in healthy human subjects. The primary endpoint is difference in EPCs between Carnosine versus placebo groups at 6 weeks and 12 weeks.

## Secondary Objective

The secondary objective of this study is to determine whether taking carnosine as a dietary supplement is protective from $PM_{2.5}$ air pollution-induced arterial dysfunction, endothelial damage, and pathological platelet activation in healthy human subjects. The secondary endpoints are difference in arterial stiffness, endothelial microparticles, and platelet monocyte aggregates between Carnosine versus placebo groups at 6 weeks and 12 weeks.

## Hypothesis (for Primary and Secondary Objectives)

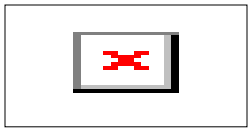
Carnosine supplementation blunts adverse effects of $PM_{2.5}$ pollution on vasculature of healthy participants.

# 7. BACKGROUND

## Rationale

Exposure to particulate air pollution has been found to be robustly associated with increased risk of cardiovascular disease (CVD) and CVD mortality. Acute exposure to fine particulate matter ($PM_{2.5}$) has been linked to adverse cardiovascular events such as myocardial infarction, stroke, cardiac arrhythmias, and sudden cardiac death, whereas chronic exposures are associated with an increased burden to CVD risk factors, insulin resistance and accelerated progression of atherosclerotic disease. However, the mechanisms by which $PM_{2.5}$ induces cardiovascular injury remain unclear and the pathophysiological conditions and co-exposures that determine individual susceptibility to $PM_{2.5}$ toxicity have not been identified. Although reductions in PM levels have been found to reduce CVD mortality, no effective individual interventions are available to mitigate the adverse health effects of $PM_{2.5}$. To design and test such interventions, it is important to understand not only the mechanisms underlying PM toxicity, but also to identify pre-existing conditions and co-exposures that moderate the cardiovascular effects of PM, so that appropriate, mechanism-based interventions can be devised and targeted to those individuals who are susceptible to the harmful cardiovascular effects of $PM_{2.5}$.

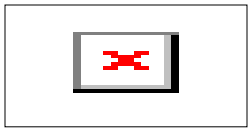
Our previous work has shown that in young, healthy adults, exposure to $PM_{2.5}$ is associated with inflammation and subclinical vascular injury. In addition, we have found that exposure to $PM_{2.5}$



decreases levels of EPCs that could potentially compromise vascular repair. We have obtained similar results in animal models, which show that exposure to concentrated ambient particles depletes EPCs, and results in defective hematopoiesis. In pilot experiments, we have found that $PM_{2.5}$-induced defects in angiogenic cell function could be prevented by treating mice with the endogenous, naturally occurring dipeptide - carnosine, which quenches reactive oxygen species and removes toxic products of lipid peroxidation. We have also found that individuals with high CVD risk are more susceptible to the effects of $PM_{2.5}$ than are low-risk individuals. Our studies also show that individuals with high levels of carnosine (a natural dipeptide) are relatively impervious to the adverse vascular effects of PM2.5. Building upon these observations, we now plan to identify individual risk factors that determine cardiovascular susceptibility to $PM_{2.5}$, and to conduct a clinical trial to test the hypothesis that in the presence of CVD risk factors, exposure to $PM_{2.5}$ induces vascular dysfunction and injury, and that these could be prevented by carnosine, which removes toxic products generated by $PM_{2.5}$ exposure. To test this hypothesis, we will assess the impact of $PM_{2.5}$ on vascular function in individuals with mild-to-moderate CVD risk, identify susceptibility factors and co-exposures that predispose individuals to PM toxicity, and determine whether the vascular effects of PM could be prevented by carnosine supplementation.

Extensive epidemiologic data show that exposure to particulate air pollution is associated with adverse health outcomes and excessive mortality.[3-5] The World Health Organization (WHO) estimates that exposure to air pollution is linked to 6.5 million deaths (approximately 12% of total deaths) per year worldwide. The pervasiveness of the health impact of air pollution is underscored by data showing that 92% of the world's population lives in areas where the levels of fine particulate matter ($PM_{2.5}$) exceed the WHO recommended annual mean concentration limit of 10$\mu g/m^3$. Thus, exposure to air pollution is a major global health threat, and is likely to remain so in future. Assessments of the impact of $PM_{2.5}$ and understanding of how $PM_{2.5}$ affects health and how these effects could be mitigated are, therefore, priority research areas of high scientific and social significance.

Although exposure to $PM_{2.5}$ increases the risk of several diseases, most (70-80%) of the excessive mortality associated with PM is attributable to CVD.[6-8] Short-term elevations in $PM_{2.5}$ levels increase the incidence of myocardial infarction, arrhythmias, stroke, and CVD mortality. In some estimates, chronic exposure to 10 $\mu g/m^3$ $PM_{2.5}$ is associated with an 8-18 % increase in CVD mortality.[9] In humans, as well as animal models,[10-12] chronic exposure to $PM_{2.5}$ is associated with increased blood pressure, progressive atherosclerotic disease, and worsening heart failure. Given the marked cardiovascular toxicity of $PM_{2.5}$, the health burden of $PM_{2.5}$ could not be substantially decreased without diminishing its cardiovascular impact. Accordingly, our studies are focused on understanding the cardiovascular effects of $PM_{2.5}$, and how they could be mitigated. Our previous work has shown that exposure to $PM_{2.5}$ decreases the levels of EPCs and that these changes are accompanied by an increase in the levels of circulating endothelial microparticles, immune cells, and anti-angiogenic cytokines[13-16]. These findings provide new insights into the nature of $PM_{2.5}$-induced cardiovascular injury, and identify new



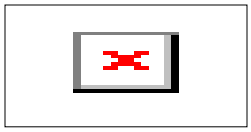
targets and biomarkers of $PM_{2.5}$ injury. Based on these findings, we now plan to elucidate the mechanistic link between $PM_{2.5}$ exposure and cardiovascular injury. Elucidation of such mechanisms is important not only for a better understanding of $PM_{2.5}$ toxicity, but also for developing mechanism-based interventions to mitigate the adverse effects of $PM_{2.5}$.

Despite its high disease burden, no effective individual-level interventions are currently available to prevent the adverse cardiovascular effects of $PM_{2.5}$. Although the deleterious effects of $PM_{2.5}$ could be attenuated by decreasing its ambient levels, such reductions are difficult, even in developed countries, due to difficulties associated with reducing traffic, or changing industrial and agricultural practices. Such societal changes take many years. Meanwhile, millions across the world are exposed to unhealthy levels of $PM_{2.5}$. Hence, effective interventions are urgently needed to lessen the adverse health effects of $PM_{2.5}$. Such interventions, even if partly successful, could significantly reduce mortality worldwide. However, attempts to develop effective interventions are thwarted by a paucity of mechanistic understanding of $PM_{2.5}$ toxicity, which is a pre-requisite for designing effective interventions that target key steps in the sequelae of $PM_{2.5}$ injury.

In addition to being mechanism-based, such interventions must also target specific populations. It has long been suspected that not all individuals are equally sensitive to $PM_{2.5}$. Indeed, some studies have reported that individuals of low socio-economic status, or those with diabetes or advanced age are particularly sensitive to $PM_{2.5}$,[17-19]but others have reported contradictory results.[20] Overall, the literature is sparse and inconsistent, and many studies are limited by the lack of information available on CVD risk factor burden, which might partly account for variability in susceptibility, which could also stem from co-exposures to other pollutants.[20] Hence, we will identify susceptible states and moderating co-exposures that determine vulnerability to $PM_{2.5}$, and delineate their contribution to cardiovascular harm associated with $PM_{2.5}$. These findings would not only lead to a better understanding of the cardiovascular pathways affected by $PM_{2.5}$, but also aid in targeting interventions to mitigate PM toxicity. Hence, the results of our project to understand the mechanisms that contribute to the cardiovascular effects of PM, to identify and validate indices and biomarker of PM-induced vascular injury, and then to test whether targeted intervention to these processes could attenuate PM associated changes address key knowledge gaps in this area and move the field forward. Additionally, findings of our project could also lead to the development of clinical guidelines for preventing $PM_{2.5}$ toxicity in susceptible individuals, and evidence-based advisories to mitigate the effects of $PM_{2.5}$ by avoiding co-exposure and protecting vulnerable populations.

## Background Pre-Clinical

In order to test whether carnosine supplementation will attenuate the effects of $PM_{2.5}$; however, to obtain proof-of-principle data for this Aim, we employed a preclinical mouse model to determine whether carnosine supplementation would attenuate the effects of $PM_{2.5}$. In these experiments, we found that mice inhaling concentrated air particles (CAPs) for 9 days show



defects in hematopoiesis, i.e., the bone marrow of mice exposed to CAPs was populated by fewer hematopoietic stem cells.

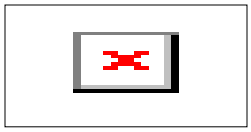
Furthermore, EPCs isolated from the bone marrow of exposed mice exhibited an impaired ability to form tubes in vitro. However, mice that were placed on carnosine-containing drinking water showed no significant defect in tube formation. To assess the physiological significance of these findings, we exposed mice to CAPs ± carnosine in drinking water. After 9 days of exposure, EPCs were isolated from these mice and injected into other mice subjected to hind limb ischemia. EPCs from exposed mice fed carnosine were able to stimulate blood perfusion, while those on normal drinking water did not. These observations, showing that carnosine attenuates the hematopoietic injury induced by $PM_{2.5}$, and prevents EPC dysfunction in mice; strengthen the rationale for conducting an interventional clinical study. Thus, collectively, our preliminary studies support key concepts of the project, i.e., $PM_{2.5}$ exposure in humans is associated with marked vascular injury that is moderated by pre-existing CVD risk and that carnosine levels and that carnosine supplementation blunts adverse effects of $PM_{2.5}$.

Vascular homeostasis and endothelial health are thought to be maintained in part by a subpopulation of pro-angiogenic cells - the EPCs that reside in the bone marrow and circulate in the peripheral blood. [21] It has been shown that upon hypoxia or vascular injury, EPCs are mobilized from the bone marrow and home to the site of tissue damage. At these sites, EPCs contribute to angiogenesis either through terminal differentiation into mature endothelial cells or by paracrine stimulation of wound healing processes. [22]

## Background Clinical

Exposure to $PM_{2.5}$ has been found to be associated with an increase in blood pressure and vascular dysfunction.[23] Our studies have shown that $PM_{2.5}$ exposure is associated with changes in several biomarkers of vascular injury, including EPCs, endothelial microparticles, fibrinogen, platelet-monocyte aggregates as well as plasma cytokines and growth factors in a cohort of young individuals with low CVD risk.[13] However, it remains unclear whether vascular function and biomarkers of vascular injury are similarly affected in our cohort of individuals with mild-to-moderate CVD risk (Framingham Risk Score, >10). Extending our previous work with a young disease-free cohort to an older, at-risk cohort is essential to identify which specific indices of vascular function and which specific biomarkers of vascular injury are associated with $PM_{2.5}$ exposure in individuals with mild-to-moderate CVD risk, as our preliminary work shows that changes in vascular parameters vary with CVD risk. Moreover, this work will permit assessment of the relationship between $PM_{2.5}$-induced changes in function and the underlying pathophysiological changes. We will further examine their interrelationships and contribution to vascular injury.

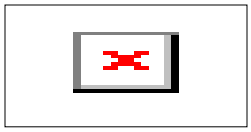
However, not all individuals are equally sensitive to $PM_{2.5}$. Previous studies have variably shown some individuals are more sensitive to $PM_{2.5}$-associated mortality than are young healthy



individuals,[18, 19, 24, 25] but the literature is sparse and conflicting.[20] Moreover, it is unclear whether changes in $PM_{2.5}$-associated vascular injury varies with individual differences in age, SES, sex, ethnicity, and pre-existing CVD risk. Our past studies have shown that $PM_{2.5}$ exposure is associated with endothelial injury even in young, healthy individuals,[13] which might be important factors contributing to progressive cardiovascular injury, hence it is important to distinguish between changes that contribute to the progression of $PM_{2.5}$-induced injury in healthy individuals, and those that heighten $PM_{2.5}$ sensitivity in individuals with high CVD risk. This distinction is important for appropriate targeting of interventions via carnosine supplementation.

In addition to individual level variables, co-exposure to other pollutants could also affect the relationship between $PM_{2.5}$ exposure and vascular injury and dysfunction. There is sparse and conflicting evidence for an association between CVD and exposure to gaseous pollutants such as ozone, oxides of nitrogen, carbon monoxide, and organic carbon (OC).[20] Identifying the role of these pollutants in epidemiological studies is difficult due to their strong correlation with each other and with PM. Moreover, epidemiological studies continue to focus on measures of OC without speciation, because of the expense, as a result, data on the effects of volatile organic compounds (VOCs) are lacking.[20] Evaluation of the role of VOCs is important, because even as the particle emission are reduced, there is increased risk of higher toxicity of remaining particles and an increase in gaseous copollutants,[26] which are expected to increase with a changing climate and by 2050, and their impact may exceed that of particulate pollution.[27] Moreover, animal data from our laboratories suggest that they cause significant cardiovascular injury.[28-32] Limited data from our group also link VOCs to CVD risk and cardiovascular injury in humans.[33] Hence, in addition to our focus on $PM_{2.5}$, we will examine the contribution of gaseous pollutants, as they are likely to affect pathways (inflammation, oxidative stress, thrombosis) similar to $PM_{2.5}$. The results of these investigations will not only provide a new understanding of the role of gaseous pollutants in inducing vascular injury, but also aid in assessing whether they heighten sensitivity to PM, which would be important in identifying individuals suitable for intervention via carnosine supplementation.

The intervention of carnosine supplementation as blinded placebo-controlled clinical trials has been extensively published in the existing literature. [34-37] Daily dose of dietary carnosine supplementation in these studies is predominantly in the range of 2 grams to 4 grams per day. A daily dietary supplementation of 2 grams carnosine has been shown to be safe and efficacious in healthy human participants.[34, 37]



# 8. INVESTIGATIONAL PLAN

## Research Questions

1. Does dietary supplementation of carnosine during a period of high $PM_{2.5}$ pollution mitigates effects on vascular indices?
2. Does dietary supplementation of carnosine during a period of high $PM_{2.5}$ pollution improve the arterial function?
3. Does dietary supplementation of carnosine during a period of high $PM_{2.5}$ pollution decrease endothelial damage?
4. Does dietary supplementation of carnosine during a period of high $PM_{2.5}$ pollution decrease platelet activation?

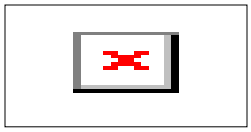
## Study Design

This is a randomized, double-blind, placebo-controlled longitudinal clinical intervention study to assess whether dietary supplementation of a naturally occurring peptide in the body will mitigate the harmful effects of seasonal $PM_{2.5}$ air pollution on indices of vascular biology. A total of 240 participants will be enrolled and randomized in 1:1 ratio to receive either dietary carnosine or placebo (cornstarch). Stratification factors are sex (birth male and female), age (22-44; 45-65) and carnosine level (very low, low). A block (or size 6) stratified randomization scheme will be used. Accounting for a conservative attrition rate of 40% (failure to complete all study time-points), a total of 400 participants will be randomized to receive either dietary carnosine supplementation or placebo (cornstarch).

The mode of dietary supplementation will be oral capsules. Intervention of dietary carnosine supplementation or placebo will occur daily, for 12 consecutive weeks during a period of high $PM_{2.5}$ pollution – May through September. Due to logistical limitations, enrollment may be split over multiple years, but will occur during the same monthly period of high $PM_{2.5}$ pollution (May through September) in the study region (30 miles radius from Louisville Kentucky). The 12 weeks supplementation period was selected based upon previous dietary carnosine supplementation trials as a reasonable and commonly used time frame to assess benefit and risk.[34, 37] Based on existing literature, a measureable effect of carnosine on many indices is noticeable by 6 weeks of starting supplementation and can reach peak effect by 12 weeks. [34, 37]

The general study enrollment and investigational plan for Year # 1 (2020) and Year # 2 (2021) is illustrated and outlined in Figure 1 and Figure 2 respectively. Details regarding each individual study visit are illustrated and outlined in Figures 3-7. The study visits and details regarding each visit are further detailed in section 10 “Study Visits”.

The study PI and other investigators will be blinded to the allocation of participants to either carnosine or placebo. The participants will also be blinded to their allocation. The Randomization Committee (RC) will be in possession of the randomization key so that accurate unblinding of the randomization assignment may be identified in the event of a medical or other



emergency. The RC will consist of four individuals: the pharmacist compounding the supplement (Dr. Peter Rice), the study physician (Dr. Andrew DeFilippis), a member of the UofL Clinical Trials Unit, and the study biostatistician (Dr. Shesh Rai). Study supplements (carnosine or placebo) will be prepackaged into punch cards and marked with letters “A” or “B”. The contents of series “A” or “B” will be part of the blinded randomization and will be known only to the RC. The study supplements will be directly shipped, stored and handled by the UofL CTU.

An initial screening (Visit # 1) of potential / interested participants will be conducted to identify eligible candidates based on eligibility criteria established *a priori*. Eligible candidates will be invited to a complete baseline assessment (Visit # 2). Randomization and allocation will occur between Visit # 1 and Visit # 2. Participants invited for Visit # 2 will already be randomized into one of the two study groups (carnosine or placebo). Participants from both study groups will be followed up at 6 weeks (Visit # 3) and 12 weeks (Visit # 4). Relevant and extensive data for primary and secondary endpoints will be collected at Visit # 2 through Visit # 4. The data collected throughout all visits (# 1 through # 4) may be used to generate preliminary data for future studies.

Visit # 1 will focus on consenting the participant for the entire study, collect contact/demographic information, and collect a urine sample. Safety assessments will be conducted at Visit # 3 and Visit # 4. Participants may be contacted for up to 12 months after enrollment via phone/email/mail for safety, adverse events, and or general follow up questions.

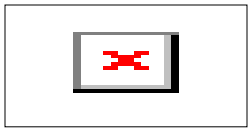
## Study Endpoints

### *Primary Endpoints (reflecting change from baseline to 6 weeks and 12 weeks and between groups)*

1. Difference in EPC-2 between Carnosine and Placebo Groups
2. Difference in EPC-3 between Carnosine and Placebo Groups
3. Difference in EPC-4 between Carnosine and Placebo Groups
4. Difference in EPC-7 between Carnosine and Placebo Groups
5. Difference in EPC-8 between Carnosine and Placebo Groups

### *Secondary Endpoints (reflecting change from baseline to 6 weeks and 12 weeks and between groups)*

1. Difference in Arterial Stiffness between Carnosine and Placebo Groups
2. Difference in Endothelial Microparticles between Carnosine and Placebo Groups
3. Difference in Platelet Monocyte Aggregates between Carnosine and Placebo Groups



## Data Collection Tools

### *Blood Samples*

A sample of ~3 table spoons (~45 ml) of blood will be obtained by venipuncture performed by a study staff trained in phlebotomy using standard procedures and precautions to insure the safety of the participants and phlebotomist. If any subject becomes ill, uncomfortable, or desires to withdraw, he/she may do so at any time. Blood sample collection protocol is detailed in Appendix A. Samples will be promptly transported to the Diabetes and Obesity Center's research laboratory on the 4th floor of the Delia Baxter building (580 S. Preston Street) for processing, storage, and analysis. After processing, samples will be aliquoted in appropriately labeled vials and stored in (-80C) freezer. Stored samples will contain study name, participant ID, date of sample acquisition, type of sample, and visit type. Samples will be stored in de-identified fashion and participant personal information or PHI will not be displayed/barcoded on any stored sample. Samples will be stored for future use, ancillary studies, and or preliminary data development.

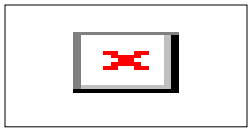
The measurement of blood biomarkers for primary and secondary endpoints from blood samples (EPCs, microparticles, and PMA) are further detailed in Appendix B.

### *Urine Samples*

Standard clean catch urine sample will be obtained from participants and stored at 4°C. Urine sample collection protocol is detailed in Appendix A. The samples will be transported on ice to the Diabetes and Obesity Center's research laboratory on the 4th floor of the Delia Baxter building (580 S. Preston Street) for processing, storage and mass spectrometric analysis. After processing, samples will be aliquoted in appropriately labeled vials and stored in (-80C) freezer. Stored samples will contain study name, participant ID, date of sample acquisition, type of sample, and visit type. Samples will be stored in de-identified fashion and participant personal information or PHI will not be displayed/barcoded on any stored sample. Samples will be stored for future use, ancillary studies, and or preliminary data development.

### *Arterial Stiffness Test (Vascular Function)*

To assess CVD risk, we will acquire arterial stiffness measures (augmentation index; AI, augmentation pressure; AP, and pulse wave velocity; PWV), aortic systolic pressure and SEVR derived from pulse wave analysis using SphygmoCor, which provides highly reproducible values.[38-40] The participant will be asked to provide the initials of their first and last name and date of birth. The device will also store the participant ID, date, and time of data acquisition. These parameters are necessary to calculate age and for data retrieval. Data will be stored on the device and other HIPAA compliant storage databases.



Measures of arterial stiffness are positively and robustly associated with increased risk for CV events, [41] and are associated with atherosclerosis, hypertension, stroke, Alzheimer-type dementia, and kidney disease. They provide novel and clinically-relevant information beyond that provided by standard risk factors; have been increasingly incorporated into longitudinal cohort studies, [41] and are particularly sensitive to air pollution exposure. [42].

SphygmoCor Xcel is a class-II FDA approved device used in clinical and research settings. SphygmoCor is a non-invasive, cuff-based tool used for obtaining brachial artery blood pressure and the central arterial pressure waveform with associated indices (augmentation index, augmentation pressure, and pulse wave velocity). A cuff will be applied on the upper arm in the standard position. The cuff will be partially inflated to record the brachial waveforms. These waveforms are detected by sensing changes in the pressure inside the cuff related to arterial pulsation. The ascending aortic waveform is subsequently derived using a validated mathematical transfer function.

The participant may be provided with their current blood pressure and heart rate, as measured by this FDA approved device.

## *Questionnaires*

Self-reported interview-style questionnaires are quick, convenient and reliable source of research data collection. This modality is used by many large NIH funded research studies like the Multi-Ethnic Study of Atherosclerosis (MESA), the National Health and Nutrition Examination Survey (NHANES), and many of our group's other NIH funded studies – the Green Heart Project, Cardiovascular Injury in Tobacco, Louisville Healthy Heart Study, etc. We will utilize these existing questionnaires (internal and external) to compile a list of relevant questions which will help in high quality data acquisition. The questionnaires will collect data on medical history, medications, environmental pollution exposure, sleep, diet, exercise, tobacco, and women's health.

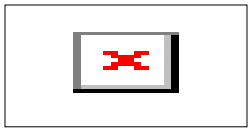
Data will be recorded in real-time in a REDCap database created specifically for this study. Since, these questionnaires will collect multiple personal information and PHI, only trained research personnel with relevant reason will be granted access.

## *Physical Examination*

Physical examination will be conducted to record the participant's height, weight, and waist circumference. Blood pressure and heart rate measurements will be provided during the arterial stiffness measurement.

## *Physical Function Test*

To assess the impact of carnosine supplementation on physical performance during times of variable $PM_{2.5}$ levels, we will utilize three physical performance tests: handgrip



strength test, 2-minute step test (2MST), and bilateral calf-raise test. The protocol for these measurements are further detailed in Appendix E.

## *Sample Size Computations and Assumptions*

The primary outcome measures are EPC-2, EPC -3, EPC -4, EPC -7, and EPC -8. Also, there are many secondary outcome measures. Based on our pilot study, mean and SD (standard deviation) are reported below.

| | EPC-2 | EPC-3 | EPC-4 | EPC-7 | EPC-8 |
|---|---|---|---|---|---|
| Mean | 0.675783 | 0.016016 | 0.050813 | 0.297092 | 0.631728 |
| SD | 0.702561 | 0.010805 | 0.056709 | 0.412414 | 0.691299 |

This is a longitudinal study and primary observations will be made at baseline, 6 weeks and 12 weeks follow-ups. Due to 5 primary outcome measures, we adjusted alpha=0.01 (=.05/5). Due to multiple sub-studies, we plan to enroll a large evaluable cohort of size n=240. With a correlation of 0.5 on any two consecutive time-point measures, (this is a conservative estimate) and n1=120, n=120 at alpha= 0.01 and power=0.90, we can detect the effect size of 0.5 SD using a two sample t-test in a longitudinal setting. The detectable effects for each EPC is given below.

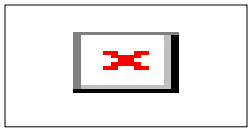
| Power | Correlation | EPC-2 | EPC-3 | EPC-4 | EPC-7 | EPC-8 |
|---|---|---|---|---|---|---|
| 80% | 0.50 | 0.351 | 0.005 | 0.028 | 0.206 | 0.346 |
| 90% | 0.50 | 0.223 | 0.003 | 0.018 | 0.131 | 0.219 |
| 80% | 0.80 | 0.197 | 0.003 | 0.016 | 0.116 | 0.194 |
| 90% | 0.80 | 0.156 | 0.002 | 0.013 | 0.092 | 0.153 |

To adjust for attrition/drop out we have increased the sample size to 400.

Associations of $PM_{2.5}$ exposures with measured outcomes will be estimated using mixed effects models. We will use interaction variables to detect whether the association between $PM_{2.5}$ and outcomes is significantly different between with or without carnosine groups. We will perform a formal mediation analysis, which allows for interactions to determine the degree to which carnosine levels mediate the effect of $PM_{2.5}$ on our outcomes. Plots of subject-specific differences over pollution concentrations along with regression plots will be generated.

## *Sample Size Recommendation*

Sample size of 240 participants (120 carnosine supplementation, 120 placebo group) provides sufficient sample size for the primary endpoint as well as other secondary endpoints.



Due to the prolonged intervention period (12 weeks), two follow-up visits and the inconvenience of taking multiple oral capsules daily, we anticipate a 40% attrition from screening (Visit # 1) through 12-week follow-up (Visit # 4). Therefore, we have established a goal of stratified identification of a total of 400 participants (1:1) at screening (Visit # 1). We anticipate that with a conservative estimate of 60% retention, 120 participants in each group (supplementation, placebo) will complete the entire study.

### *Multiple Testing Issue*

Type I error correction in the context of an early phase clinical trial that performs multiple tests can serve to protect the study interpretation against Type I error inflation. However, the use of corrections for multiple comparisons in “proof-of-concept-studies” is problematic. At this investigational level, tight control of the overall family-wise Type I error rate would increase the likelihood that the investigators would miss a finding of potential importance by attributing it to chance simply because the p-value is large. The study investigators have, in this protocol, attempted to balance the need to control the number of evaluations with the need to identify new effects by not incorporating a multiplicity correction. The primary outcomes measures will be declared significant for alpha <0.01. There will not be any adjustments for secondary outcome measures and will be declared significant at alpha <0.05.

# 9. STUDY ENROLLMENT AND WITHDRAWAL

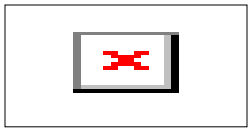
## Introduction

Potential subjects will be males and females, all ethnicities, 22-65 years of age, living within or near the Louisville metropolitan area, with carnosine levels below the median value of the population.

## Criteria to Invite for Screening Visit (Visit # 1)

To be considered eligible to participate in the screening visit of the study, the participant must meet the following criteria:

1. Living within 30 miles radius of Louisville Metropolitan area (Kentucky counties: Jefferson, Bullitt, Spencer, Shelby, and Oldham; Indiana counties: Clark, Floyd).
2. Age 22-65 at time of baseline assessment (Visit # 2)
3. Healthy subjects
4. Subjects with a diagnosis that puts them at high risk for acute or chronic disease, specifically but not limited to hypertension or diabetes, are eligible for the study if the condition is treated by a physician or provider and is controlled via medications and or lifestyle that are otherwise not excluded by the exclusion criteria.
5. Consumes some type of meat/fish at least once a month during the past 3 months



6. Agree to complete all four study visits (one screening, one baseline, two follow-up visits)
7. Will be living in the Louisville Metropolitan area throughout the study period (May through September), with planned vacations of no more than 2 weeks away from study area.
8. Agree to take supplement/placebo intervention during the study period and follow study intervention regimen

## Main Study Inclusion Criteria (Visits # 2-4)

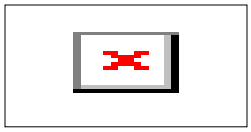
To be considered eligible to participate in this study, the participant must meet the following criteria:

1. Living within 30 miles radius of Louisville Metropolitan area (Kentucky counties: Jefferson, Bullitt, Spencer, Shelby, and Oldham; Indiana counties: Clark, Floyd).
2. Age 22-65 at time of baseline assessment (Visit # 2)
3. Consumes some type of meat/fish at least once a month during the past 3 months
4. Carnosine level less than median (value of the population
5. Agree to complete all four study visits (one screening, one baseline, two follow-up visits)
6. Will be living in the Louisville Metropolitan area throughout the study period (May through September), with planned vacations of no more than 2 weeks away from study area.
7. Agree to take supplement/placebo intervention during the study period and follow study intervention regimen

## Exclusion Criteria (All Visits)

Participants will be excluded from the study if they have any of the following conditions:

1. Consumed any carnosine supplement of more than 6 grams per a week in the past 4 weeks (one month)
2. Current / ongoing treatment for substance abuse
3. Currently undergoing treatment or have conditions which may cause participant to be immunosuppressed
4. Diseases Affecting Peripheral Cell Count (i.e. Autoimmune Diseases – Hashimoto, Rheumatoid Arthritis, SLE, Rheumatoid Arthritis, Sjogren syndrome, Ankylosing Spondylitis, Takayasu arteritis, Kawasaki disease, Polyarteritis nodosa.)
5. Diseases Affecting Bone Marrow capacity
6. Diagnosis of any active cancer
7. Recent organ / kidney transplant or replacement (Active/Long-Term Medications)
8. Type 1 Diabetes Mellitus
9. Untreated thyroid disease
10. Untreated anemia
11. Current acute infections (Influenza, fever, etc.)
12. HIV positive status
13. Active/current Hepatitis HepA, HepB or HepC or in past 6 months
14. Pregnant / lactating or planning to



15. Prisoners / vulnerable populations
16. Other medical conditions that compromise completion of study
17. Unwilling to provide consent

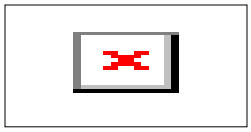
## Special Conditions of Exclusion (Screening or Main Study)

Candidates with the following conditions or history may be excluded:

1. **Local reactions**: diarrhea, stomach upset, tingling sensation in extremities, rash or itchiness, dry mouth, changes in appetite, feelings of tiredness, or vivid dreams.
2. **Systemic reactions**: fever, allergic reaction, anaphylaxis or any clinical untoward event that occurred with a known cause of taking dietary supplement.
3. **Abnormal laboratory results** which are deemed clinically significant with a known cause of taking dietary supplement.
4. **Pregnancy and breast-feeding**: Currently, there is not enough reliable information about the safety of taking carnosine supplement during pregnancy or breast-feeding. Hence, participants/candidates who are currently pregnant or breast-feeding or plan to be pregnant/breast-feeding during the study period will not be enrolled in the study.
5. **Low blood pressure**: Carnosine synthesized in the body has been found to decrease blood pressure via influencing the autonomic nervous system. [43] Hence, in theory, taking supplemental carnosine might lower blood pressure and or exacerbate lowering of blood pressure in people taking antihypertensive medications. Hence, all participants taking blood pressure lowering medication will be cautioned for this interaction and advised that in case of an episode of low blood pressure to discontinue study supplement and immediately seek medical attention from a licensed healthcare provider.
6. **Low blood sugar**: Carnosine synthesized in the body has been found to decrease blood sugar via influencing the autonomic nervous system. [43] Hence, in theory, taking supplemental carnosine might lower blood sugar and or exacerbate lowering of blood sugar in people taking blood sugar lowering medications. Hence, all participants taking blood sugar lowering medication will be cautioned for this interaction and advised that in case of an episode of low blood sugar to discontinue study supplement and immediately seek medical attention from a licensed healthcare provider.

## Prescreening (Prior to Consent)

Prescreening participants includes reviewing personal or protected health information for inclusion/exclusions prior to consent. Also, currently we have a database of about 1,000 subjects who have previously participated or contacted us for participating in one of our research studies and expressed interest in participating in future research studies. Only candidates who have given permission to contact them for future studies will be prescreened for relevant eligibility criteria and contact information. The script for phone and email/letter communication is detailed in Appendix C.



## Consent

All participants enrolled in this clinical trial will be identified as males or females, age 22-65, living within 30 miles radius of Louisville metropolitan area, and with carnosine levels less than the median of local population. After being identified (from existing database, from clinics, flyers, or other routes), potential participants will be approached by one of the investigators or research coordinators. The information provided to the potential participant is included in the informed consent. The informed consent will provide information regarding $PM_{2.5}$ pollution, prevalence of $PM_{2.5}$ pollution in the study area, synthesis and natural role of carnosine in the body, natural and supplemental sources of carnosine, cornstarch (constituent of placebo), and will include information regarding possible risks of participation.

## Randomization

Eligible participants identified from Screening (Visit # 1) will be randomized in to supplement or placebo groups by 1:1 distribution. Randomization will be performed in accordance with written standard operating procedures by personnel that are not involved in the selection, screening, or enrollment of subjects and blinded as to study documentation. Randomization to supplement assignment will be conducted using a web access database created and maintained by the Randomization Unit. After the investigator or research coordinator verifies that screening testing is complete and the inclusion and exclusion criteria for the study have been satisfied, an un-blinded member of the Randomization Unit will have secured access to the participant's supplement assignment. The participant will be randomized a minimum of 2 days before the scheduled date of the baseline assessment (Visit # 2) to arrange for the timely availability of the study supplement. See Manual of Operations for details

## Stopping Early or Withdrawal from the Study

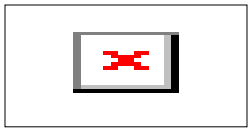
Monitoring rules and statistical boundaries for adverse outcomes are further detailed in Section 15 "Data and Safety Monitoring Board". Below is the outline for recusal or withdrawal of participant from the study due to a non-health / safety related reason.
Participants that have consumed study supplement for at least 4 weeks during the duration of the study period, but missed taking the study supplement for no more than 1 week during the study period will still be followed if feasible, and included in intent to treat analysis.

Participants that withdraw from the study before Follow-Up # 1 (Visit # 3) or do not complete Visit # 3 will be considered lost to follow-up. Existing data of these participants will be retained for further analysis.

## Screen Failure

Candidates are considered screen failures when they meet one of the following criteria after signing consent:

1. Screening (Visit # 1) data reveals that the candidate is ineligible
   OR



2. Candidate withdraws and does not complete Baseline Assessment (Visit # 2)

## Discontinued

Participants are considered discontinued when they meet one or more of the following criteria:

1. Participant withdraws consent after being randomized
   OR
2. Participant withdraws consent after completion of Baseline Assessment (Visit # 2)
   OR
3. Participant fails to follow study supplement regimen
   OR
4. Participant is unable to fulfill the inclusion/exclusion criteria during the study
   OR
5. Participant refuses/is unable to provide critical data at follow-up visits as deemed necessary by the investigator
   OR
6. Participant is withdrawn after randomization by investigator for reasons that may compromise completion of the study

## Medical or Health Concerns

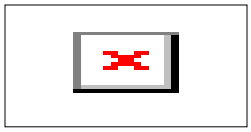
Participants will be instructed that in an event of an acute health concern (related or unrelated to the study supplement), they should immediately discontinue the study supplement and seek medical attention from a licensed healthcare provider. Participants will be provided with a study identification card. This card will contain the following information: name of the study, name of study institution, a statement to the effect of "*each capsule may contain 0.5 grams of L-Carnosine or 0.5 grams of cornstarch*", method of contacting the study physician, and a method of contacting the study coordinator.

Participants may be able to re-start supplement regimen and continue the randomization after detailed discussions with the study physician. The study physician may seek advisement from the DSMB on these matters.

## Compensation

Participants will be given a University of Louisville branded prepaid VISA gift card for participating in the study. The following information will be collected: full name, address, and social security number. Social security number will not be saved. As required by the Internal Revenue Service, participants may need to report the payment as income on their taxes. If participants choose not to provide their social security number or other required information, they will not be paid for participating in the study. Participants can still participate in the study even if they don't want to be paid.

Participants will be compensated for completing a clinical visit as per the following schedule:



| Visit Description | Amount |
|---|---|
| Screening (Visit # 1) | $25 |
| Baseline Assessment (Visit # 2) | $50 |
| Follow-Up # 1 (Visit # 3) | $75 |
| Follow-Up # 2 (Visit # 4) | $100 |
| 4-12 Month Safety Check (Visit # 5)* | None |

*Visit # 5 is not an actual physical visit, contact will be made via phone, email, or mailed paper survey

# 10. STUDY VISITS

## Screening (Visit # 1)

The screening period extends from the date informed consent is signed until the day of randomization (Figure 3). The screening window will not exceed 180 days prior to randomization.

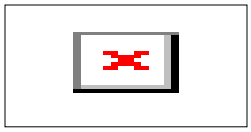
The following activities will be carried out at screening:

1. Informed Consent (for entire study)
2. Blood and or Urine sample collection
3. Demographic data
4. Contact information
5. Prepaid $25 VISA gift card

Participants may be excluded from the study if any of the above screening data collection is not completed or meets an exclusion criterion during the screening period. Data gathered from screening (Visit # 1) will be used to identify whether the candidate meets the enrollment criteria for the main study and whether they are eligible to be randomized. Eligible candidates will be invited to participate in the main study and subsequently randomized in a double blind fashion.

## Baseline Assessment (Visit # 2)

Baseline assessment will occur after eligible candidates have been identified via screening (Visit # 1) (Figure 4). Participants attending the baseline assessment (Visit # 2) will already be randomized into carnosine or placebo group. Participants will be provided with the study supplement during this visit and they will start their dietary supplement regimen (carnosine or placebo) immediately on the day of baseline assessment. The following activities will be carried out at Baseline Assessment:



1. Update Demographics and Contact Information
2. Questionnaire data on Medical History, Medications, Exposure to Environmental Pollution, Sleep, Diet, Exercise, Tobacco, and Women's Health
3. Blood sample collection (~3 tablespoons or ~45ml)
4. Urine sample collection (~2 tablespoons or ~30ml)
5. Arterial stiffness test (SphygmoCor)
6. Physical strength test (hand grip test, step test, calf raise test)
7. Provide 8 weeks of study dietary supplement capsules (carnosine or placebo)
8. Discuss schedule and related instructions related to taking the study dietary supplement
9. Prepaid $50 VISA gift card

Participants may be excluded from the study if any of the above data collection is not completed or meets an exclusion criterion during this period.

## Follow-Up # 1 (Visit # 3)

Follow-Up # 1 will occur ~6 weeks after initiation of study supplement at the baseline assessment (Visit # 2) (Figure 5). Participants attending follow-up # 1 (Visit # 3) will have already been taking the study supplement for at least 5 weeks and no more than 7 weeks (6 weeks $\pm$ 1 from Visit # 2). The following activities will be carried out at Follow-Up # 1:

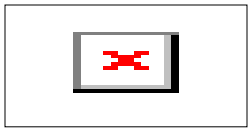
1. Update Demographics and Contact Information
2. Update Questionnaire data on Medical History, Medications, and Women's Health
3. Questionnaire data Exposure to Environmental Pollution, Sleep, Diet, Exercise, Tobacco
4. Blood sample collection (~3 tablespoons or ~45ml)
5. Urine sample collection (~2 tablespoons or ~30ml)
6. Arterial stiffness test (SphygmoCor)
7. Physical strength test (hand grip test, step test, calf raise test)
8. Review the participant's dietary supplement regimen and any instances of deviation in regimen (missed or over consumption).
9. Provide 4 more weeks of study dietary supplement capsules (carnosine or placebo)
10. Discuss any side-effects which may have occurred or may arise from study supplement. Discuss any health changes during the study period.
11. Prepaid $75 VISA gift card

Participants may be excluded from the study if any of the above data collection is not completed or meets an exclusion criterion during this period.

## Follow-Up # 2 (Visit # 4)

Follow-Up # 2 will occur ~12 weeks after initiation of study supplement at the baseline assessment (Visit # 2) (Figure 6). Participants attending follow-up # 2 (Visit # 4) will have already been taking the study supplement for at least 10 weeks and no more than 12 weeks (11 weeks $\pm$ 1 from Visit # 2). The following activities will be carried out at Follow-Up # 2:

1. Update Demographics and Contact Information



2. Update Questionnaire data on Medical History, Medications, and Women's Health
3. Questionnaire data Exposure to Environmental Pollution, Sleep, Diet, Exercise, Tobacco
4. Blood sample collection (~3 tablespoons or ~45ml)
5. Urine sample collection (~2 tablespoons or ~30ml)
6. Arterial stiffness test (SphygmoCor)
7. Physical strength test (hand grip test, step test, calf raise test)
8. Review the participant's dietary supplement regimen and any instances of deviation in regimen (missed or over consumption).
9. Discuss any side-effects which may have occurred or may arise from study supplement. Discuss any health changes during the study period.
10. Prepaid $100 VISA gift card

Participants may be excluded from the study if any of the above data collection is not completed or meets an exclusion criterion during this period.

## 4 – 12 Month Safety Check (Visit # 5)

**Although this interaction is termed as a "Visit", it will not involve participants to physically be present**. A follow-up email / phone call / mailed paper questionnaire will be conducted 4-12 months after the end of supplement intervention (Figure 7). This contact will focus on updating about any SAEs & medical / medication history changes since end of intervention:

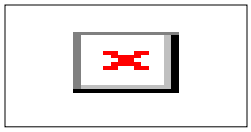
1. Update Demographics and Contact Information
2. Questionnaire data on Medical / Medications History since end of intervention
3. Questionnaire data on adverse effects or SAEs since end of intervention.
4. No monetary compensation for this follow-up / check.

Participants will not be excluded from the study if any of the above data collection is not completed. Instead, they will be categorized as "lost to safety follow-up".

## Safety Evaluations during Study Visits

Adverse events will be assessed during Visit # 3 and Visit # 4 and through the 12 months after participants have stopped taking study supplement. The following will be documented in the adverse event reporting system:

1. **Local reactions**: diarrhea, stomach upset, tingling sensation in extremities, rash or itchiness, dry mouth, changes in appetite, feelings of tiredness, or vivid dreams.
2. **Systemic reactions**: fever, allergic reaction, anaphylaxis or any clinical untoward event that occurs within 30 days of beginning study supplement.
3. **Abnormal laboratory results** which are deemed clinically significant.
4. **Pregnancy and breast-feeding**: Currently, there is not enough reliable information about the safety of taking carnosine supplement during pregnancy or breast-feeding. Hence, participants/candidates who are currently pregnant or breast-feeding or plan to be pregnant/breast-feeding during the study period will be "discontinued" from the study.



5. **Low blood pressure**: Carnosine synthesized in the body has been found to decrease blood pressure via influencing the autonomic nervous system. [43] Hence, in theory, taking supplemental carnosine might lower blood pressure and or exacerbate lowering of blood pressure in people taking antihypertensive medications. Hence, all participants taking blood pressure lowering medication will be cautioned for this interaction and advised that in case of an episode of low blood pressure to discontinue study supplement and immediately seek medical attention from a licensed healthcare provider.
6. **Low blood sugar**: Carnosine synthesized in the body has been found to decrease blood sugar via influencing the autonomic nervous system. [43] Hence, in theory, taking supplemental carnosine might lower blood sugar and or exacerbate lowering of blood sugar in people taking blood sugar lowering medications. Hence, all participants taking blood sugar lowering medication will be cautioned for this interaction and advised that in case of an episode of low blood sugar to discontinue study supplement and immediately seek medical attention from a licensed healthcare provider.

Participants will be advised to continue / discontinue after a review by the study physician and the DSMB.

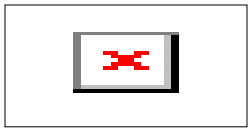
# 11. STUDY SUPPLEMENTS

Eligible participants identified from Screening (Visit # 1) will be randomized into carnosine or placebo groups by 1:1 distribution.

## Supplement Quality Control and Regulations

As per the Office of Inspector General of the Department of Health and Human Services' report on "Dietary Supplement Labels: Key Elements" – "*The Dietary Supplement Health and Education Act of 1994 (DSHEA) [44] defines the term "dietary supplement" to mean a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of any of the aforementioned ingredients*". [44, 45]

The United States Food and Drug Administration (US FDA) guidelines specify that merchants selling dietary supplements in the United States are required to follow current good manufacturing practices and their manufacturing plants are required to be inspected by the FDA for "*quality of the dietary supplement and to ensure that the dietary supplement is packaged and labeled as specified in the master manufacturing record*".[45] Dietary supplements are not regulated by the FDA for their efficacy but will be inspected for quality, safety, and accuracy.[44, 45]



Both, Carnosine and placebo, supplements will be prepared by the University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences. Commercially available L-carnosine powder and cornstarch (placebo) will be packaged into gelatin coated capsules in laboratory facilities associated with the state licensed pharmacy of the University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences.

## Supplement Capsule Preparation

Empty size 000 capsules will be purchased from www.CapsulCN.com and filled by trained individuals (technicians and Doctorate of Pharmacy students) under the supervision of a compounding pharmacist. Personnel involved in capsule preparation will be trained in but not limited to: practice and verification of capsule weight consistency. An appropriate amount of L-carnosine will be weighed, placed on a capsule packing device, and evenly distributed to each capsule. Capsules will be then sealed, locked and “polished” to remove any residual powder. Capsules will be then checked for weight consistency. USP standards allow 10% variation; variation in capsules prepared by this pharmacy is under 5% and typically less than 2%.

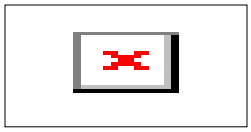
L-carnosine will be purchased in bulk from www.BulkSupplements.com. The FDA inspection registration number of www.BulkSupplements.com is 17938017996. Placebo supplements will be composed of cornstarch, and will match the composition routinely used in clinical interventional studies. Cornstarch placebo will be purchased from Medisca (Plattsburgh, NY). Further details about the materials, preparation, safety testing and other items related to supplement or placebo are detailed in Appendix D.

## Supplement Assignment

The study supplements (carnosine and placebo) are each individually described below but shall thereafter be referred to throughout the protocol as “study supplement”. In the circumstance that the participant misses taking the supplement on for less than 3 consecutive and 4 total days in the 2 weeks leading to Follow-Up # 1 (Visit # 3), the participant will continue to be followed (intent to supplement analysis).

## Carnosine Supplement

Two hundred participants will receive Carnosine supplement. Carnosine supplement will be available in forms of oral capsules coated with bovine bone derived gelatin. Each capsule will contain 0.5 grams of Carnosine supplement. The participants of the Carnosine group will be required to take a total of 2 grams of daily carnosine supplement. Participants will be required to take Carnosine supplement twice per day: 1) Morning – 2 capsules, each containing 0.5 grams of Carnosine and 2) Evening – 2 capsules, each containing 0.5 grams of Carnosine. As per manufacturer instructions, the capsules can be taken with water or any other non-alcoholic drink. There is no pre-requisite of consuming a meal prior to taking the supplement.



The following activities and documentations will be performed and saved by the University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences for the Carnosine supplement:

1. Prepare, label, package, and deliver carnosine supplement and chain of custody form to courier as appropriate,
2. Maintain Carnosine chain of custody forms, certificate of analysis forms, and accountability logs (so that clinical / recruitment staff does not have access).
3. Keep completed chain of custody forms, on site for study files and quality assurance purposes.

### *Placebo Supplement*

Placebo supplements will be composed of cornstarch, and will match the composition routinely used in clinical interventional studies. The placebo supplement will also be manufactured by the University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences.

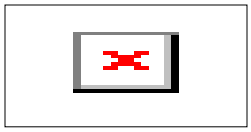
Two hundred participants will receive placebo supplement. Placebo supplement will be available in forms of oral capsules coated with bovine bone derived gelatin. Each capsule will contain 0.5 grams of cornstarch. The participants of the placebo group will be required to take a total of 2 grams of daily cornstarch. Participants will be required to take cornstarch twice per day: 1) Morning – 2 capsules, each containing 0.5 grams of cornstarch and 2) Evening – 2 capsules, each containing 0.5 grams of cornstarch. As per manufacturer instructions, the capsules can be taken with water or any other non-alcoholic drink. There is no pre-requisite of consuming a meal prior to taking the supplement.

The following activities and documentations will be performed and saved by the University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences for the placebo (cornstarch) supplement:

1. Prepare, label, package, and deliver placebo (cornstarch) supplement and chain of custody form to courier as appropriate,
2. Maintain placebo (cornstarch) chain of custody forms, certificate of analysis forms, and accountability logs (so that clinical / recruitment staff does not have access).
3. Keep completed chain of custody forms, on site for study files and quality assurance purposes.

## Randomization and Blinding

Blinding of the study personnel recruiting the participants and giving the pill-boxes will be facilitated by the fact that all study participants (Carnosine and placebo) will receive a dietary supplement. All study dietary supplements will be matched in color, consistency, and taste. The primary and secondary endpoints of the study will be determined by core laboratories whose personnel are blinded to supplementation assignment. In addition, the study recruitment and regulatory teams will take steps to ensure that endpoint and adverse event assessments are carried out in a blinded fashion.



Should unblinding occur, the Executive Committee consisting of the study PI, the study physician, and UofL CTU will be informed about the circumstances and they will discuss the circumstances related to the unblinding and further course of action. Any instance of unblinding will be documented in the study database and will be reported to the IRB as required.

## Study Supplement Requirements

### *Study Supplement Packaging and Labeling*

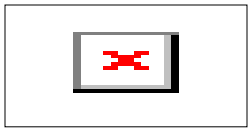
Study supplement packaging and labeling will meet all local and federal requirements. Study supplement (carnosine and placebo) label includes the product number, letter “A” or “B”, volume, expiration date and time, storage conditions, and contact name and address. All supplements will be labeled for investigational use only.

Supplement capsules will be pre-sorted into monthly (28 day) pill boxes by the unblinded laboratory personnel at the University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences. Pre-sorted pill boxes will be created for a total of 12 weeks per participant. The University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences personnel will label each box clearly as letter “A” or letter “B”, indicating whether the box contains Carnosine or placebo supplement. The study personnel at the University of Louisville involved in recruitment, processing, analysis, or any other direct participant contact will be blinded to whether “A” boxes contain Carnosine or placebo, and similarly whether “B” boxes contain Carnosine or placebo. The University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences personnel will ensure that there is absolute consistency in the contents of each series of labeled boxes. The University of Colorado Skaggs School of Pharmacy and Pharmaceutical Sciences will ensure that the contents of each box are not disclosed to anyone involved with the study (staff, investigator, or participant) and will directly and securely send the randomization key to the Randomization Committee (study physician and UofL CTU).

### *Study Supplement Storage Requirements*

L-Carnosine is tested on arrival to establish its purity and retested about every 6-12 months. L-Carnosine receives a three-year dating each time it passes re-assay. Product is stored at controlled room temperature. A sample of packed capsules will be retained, which will be re-assayed about every six months. Potency and stability testing services will be provided using Nuclear Magnetic Resonance and Liquid Chromatography/Mass Spectrometry in the Medicinal Chemistry Core Facility of the University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences.

United States Pharmacopeia (USP) <795> allows 180 day dating for compounded capsules, with extension of dating with ongoing stability studies. Initial potency and purity are established using Nuclear Magnetic Resonance and Liquid Chromatography/Mass



Spectrometry by Michael F. Wempe, PhD in the University of Colorado Skaggs School of Pharmacy, Medicinal Chemistry Core Facility. L-Carnosine (bulk powder) has up to a three-year dating from the last date of analysis. Dr. Wempe will perform ongoing stability studies to ensure that the doses we prepared remain in date throughout the study. Earlier stability studies support a dating of more than three years for our capsules.

Study supplement must be stored at room temperature from the time of manufacture until the time of giving to the study participant. The participant will be informed of these requirements and instructed to strictly follow them.

### *Study Supplement Accountability*

The study supplements will be stored in the UofL CTU, housed at Healthcare Outpatient Care Center (401 E. Chestnut Street, Suite 410, Louisville, KY, 40202). Upon receipt of study supplement from manufacturer, UofL CTU will open the shipment and verify shipment inventory, temperature, and condition of the study supplement by completing and signing the chain of custody form and forwarding the document to the unblinded manufacturing personnel. The UofL CTU personnel will also compare blinding letter (“A” or “B”). Records shall be kept of all study supplies received, disposition, and any study supplements not used or left over. Study supplement accountability logs contain unblinded information and should be maintained by the Clinical Trials Unit staff only.

The supplements accountability logs will record use of placebo as well.

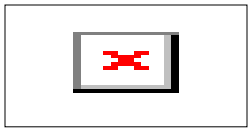
Under no circumstances will any investigator(s) allow study supplement to be used for any purpose other than that specified by the protocol. The study supplement are only to be used as a dietary supplement only for participants of this study and for their specific study group (Carnosine or placebo) only.

## Study Supplement Handling and Disposal

Destruction of unused study supplements will proceed as directed by manufacturer or designee, with appropriate documentation completed and retained by site personnel. All records involving receipt, disposition, administration, return and/or destruction of the study supplement will be made available by the PI (or designee) to the Sponsor or designee upon request.

## Taking Pre-Existing Medications

Participants can remain on their current / pre-existing medications that they were taking prior to enrollment. Data on medications added or discontinued during the course of the study will be recorded throughout the study to the 4-12 month safety check.



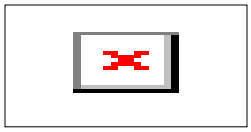
## Other Supplements

Participants may not consume any other dietary protein supplements (e.g. Whey protein drinks, Collagen powder, etc.) during the study period. Since Carnosine is an amino acid that serves as a protein building block for muscles, simultaneous consumption of protein supplement has the potential to interfere with the effects of Carnosine on muscles and body physiology.

# 12. CLINICAL AND LABORATORY EVALUATIONS

## Schedule of Procedures

| Study Activities | Screening (Visit # 1) | Screening (Visit # 2) | Screening (Visit # 3) | Screening (Visit # 4) | 4-12 Month Safety Check |
|---|---|---|---|---|---|
| Informed Consent | X | | | | |
| Contact & Demographics | X | X | X | X | |
| Medical History | | X | X | X | X |
| Medications History | | X | X | X | X |
| Physical Exam | | X | X | X | |
| Blood Samples | | X | X | X | |
| Urine Samples | X | X | X | X | |
| Arterial Stiffness Exam | | X | X | X | |
| Physical Function Exam | | X | X | X | |
| Environmental Pollution Exposure Questionnaire | | X | X | X | |
| Sleep Questionnaire | | X | X | X | |
| Diet & Exercise Questionnaire | | X | X | X | |
| Supplement Distribution | | X | X | | |
| Adverse Events Check | | | X | X | X |
| Checkout (Prepaid Visa) | X | X | X | X | |



## Procedure Details

### *Contact and Demographics*

A detailed account of the participant's contact and demographic information will be collected at screening (Visit # 1). This information will be critical while implementing the screening criteria and selecting eligible candidates for randomization. This information is also required for NIH progress report reporting. It will be administered by a research team member at every clinical study visit (Visit # 1 through # 4).

### *Participant Medical History and Physical Exam*

A complete participant history including medical, alcohol, tobacco, and medication review will be conducted at baseline assessment (Visit # 2).

A complete physical exam including vitals, height, and weight will be completed at baseline assessment (Visit # 2). Similar physical exams will be conducted at each additional clinic visit (Visit # 3 and Visit # 4) during the study.

### *Blood and Urine Samples*

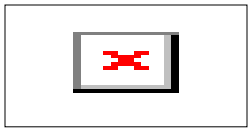
A fresh catch urine sample will be collected at screening (Visit # 1). Urine sample collection details are in Section 8 "Investigational Plan" and Appendix A. This is necessary to identify the candidate's body carnosine level and to implement the inclusion criteria. This information is critical in screening and selecting eligible candidates for randomization. A fresh catch urine sample will be collected by a research team member at every clinical study visit (Visit # 1 through # 4).

Fresh stick blood samples will be collected at baseline assessment (Visit # 2). Blood sample collection details are in Section 8 "Investigational Plan" and Appendix A. Fresh stick blood samples will be collected at each additional clinical study visit from baseline onwards (Visit # 2 through Visit # 4).

### *Arterial Stiffness Test*

SphygmoCor will be used to measure indices of arterial stiffness (augmentation index; AI, augmentation pressure; AP, pulse wave velocity; PWV, aortic systolic pressure, and SEVR).

SphygmoCor is a non-invasive, cuff-based tool used for obtaining brachial artery blood pressure and the central arterial pressure waveform with associated indices (augmentation index, augmentation pressure, and pulse wave velocity). A cuff will be applied on the upper arm in the standard position. The cuff will be partially inflated to record the brachial waveforms. These waveforms are detected by sensing changes in the pressure inside the cuff related to arterial pulsation. The ascending aortic waveform is subsequently derived using a validated mathematical transfer function.



Arterial stiffness test will be administered by a research team member at baseline assessment (Visit # 2) and then at each additional clinical study visit from baseline onwards (Visit # 2 through Visit # 4). Arterial stiffness testing (via SphygmoCor) is detailed in Section 8 “Investigational Plan”.

### *Physical Function Test*

To assess the impact of carnosine supplementation on physical performance during times of variable $PM_{2.5}$ levels, we will utilize three physical performance tests: handgrip strength test, 2-minute step test (2MST), and bilateral calf-raise test.
Physical function test will be administered by a research team member at baseline assessment (Visit # 2) and then at each additional clinical study visit from baseline onwards (Visit # 2 through Visit # 4). Testing protocol is detailed in Section 8 “Investigational Plan” and Appendix E.

### *Environmental Pollution Exposure Questionnaire*

This questionnaire will assess participant’s exposure to environmental pollution. It will be administered by a research team member at baseline assessment (Visit # 2), Follow-Up # 1 (Visit # 3) and Follow-Up # 2 (Visit # 4).

### *Sleep Questionnaire*

This questionnaire will assess participant’s sleep habits during the study period. It will be administered by a research team member at baseline assessment (Visit # 2), Follow-Up # 1 (Visit # 3) and Follow-Up # 2 (Visit # 4).

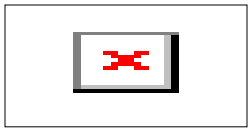
### Diet and Exercise Questionnaire

This questionnaire will assess participant’s dietary and exercise habits and their possible impacts on study supplement intake and processing throughout the study period. It will be administered by a research team member at baseline assessment (Visit # 2), Follow-Up # 1 (Visit # 3) and Follow-Up # 2 (Visit # 4).

## Storage, Genetic Testing, and Future Use of Biospecimens

Blood and urine biospecimens collected during the study (detailed in Section 8 “Investigational Plan” and Appendix A) will be processed to be stored for long period. Majority samples will be processed, aliquoted, labeled, cataloged and immediately stored in -80C freezers in the Envirome Institute’s research laboratories located on the 2nd or 3rd floors of the Cardiovascular Innovation Institute (302 E. Muhammad Ali Blvd, Louisville, KY 40202).

Samples will be used to test for current hypothesis / endpoints and or generation of preliminary data for future studies. Samples may be used to genetic testing. These collected samples will be used for research purposes only. Samples will be stored without personal identifying



information, and will be shared with approved researchers who will conduct studies to improve the scientific knowledge including but not limited to environmental pollution and cardiovascular diseases. Samples will be retained for as long as possible.

## Follow-Up Evaluations

### *Follow-Up Windows*

The timeline for follow-up will begin on the day of baseline assessment (Visit # 2). The time windows for each of the subsequent follow-up visits will be as follows:

1. Follow-Up # 1 (Visit # 3) will be 6 weeks (6 weeks ± 1 week post initiation of study supplement)
2. Follow-Up # 2 (Visit # 4) will be 12 weeks (11 weeks ± 1 week post initiation of study supplement)
3. The 12 month safety check (via phone / email / mailed paper survey) will occur between 4 months and 12 months post completion of study supplement.

### *Scheduling and Lost to Follow-Up*

Randomized participants will be followed for efficacy for 12 weeks and for safety up to one year (12 months). Participants will be considered lost to follow-up after 3 consecutive failed telephone/email contacts AND one certified letter returned to the site. Contact attempts will be documented in the participant's study chart.

# 13. ADVERSE EVENTS AND REPORTING

The definitions for adverse and related events for this study have been adapted from 21 CFR 312.32(a) and FDA guidance for industry and investigators.[46] The definitions are as follows:

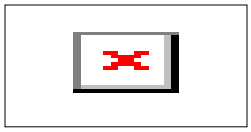
## Adverse Events (AEs)

An adverse event (AE) is defined as any untoward medical occurrence associated with the use of a supplement in humans, whether or not considered supplement related.

An AE (also referred to as an adverse experience) can be any unfavorable and unintended sign (e.g., an abnormal laboratory finding), symptom, or disease temporally associated with the use of a supplement, and does not imply any judgment about causality. An adverse event can arise with any use of the supplement (e.g., off-label use, use in combination with another drug/supplement) and with any route of administration, formulation, or dose, including an overdose.

## Suspected Adverse Reaction (SARs)

Suspected adverse reaction (SAR) is defined as any adverse event for which there is a reasonable possibility that the supplement caused the adverse event. For the purposes of safety



reporting, '*reasonable possibility*' means there is evidence to suggest a causal relationship between the supplement and the adverse event. A suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a supplement.

Within the reporting requirement under 21 CFR 312.32(c)(1)(i), the FDA provides a clear illustration of '*reasonable possibility*' by providing the following examples of types of evidence that would suggest a causal relationship between a supplement and an adverse event:

- A single occurrence of an event that is uncommon and known to be strongly associated with supplement exposure (e.g., angioedema, hepatic injury, Stevens-Johnson Syndrome)
- One or more occurrences of an event that is not commonly associated with supplement exposure, but is otherwise uncommon in the population exposed to the drug (e.g., tendon rupture)
- An aggregate analysis of specific events observed in a clinical trial (such as known consequences of the underlying disease or condition under investigation or other events that commonly occur in the study population independent of supplementation therapy) that indicates those events occur more frequently in the supplement group than in a concurrent or historical control group

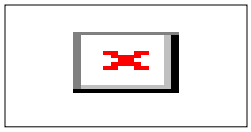
Suspected adverse reactions are the subset of all adverse events for which there is a reasonable possibility that the supplement caused the event. Inherent in this definition, and in the requirement to report suspected adverse reactions, is the need for the sponsor to evaluate the available evidence and make a judgment about the likelihood that the supplement actually caused the AE.

## Serious Adverse Events (SAEs) or Serious Suspected Adverse Reaction (SSARs)

A serious adverse event (SAE) or serious suspected adverse reaction (SSAR) is defined as an AE/SAR which, in the view of the Investigator or Sponsor, results in: 1) Death; 2) a life-threatening event (i.e. an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe); 3) inpatient hospitalization of > 24 hours or prolongation of existing hospitalization; 4) a significant disability/incapacity; or 5) a congenital anomaly/birth defect. Other important medical events may be considered SAEs/SSARs if, in the opinion of the Investigator(s), they jeopardize the participant or require intervention to prevent one of the other outcomes listed above.

## Role of Abnormal Test Findings in Classifying an Event

The tests conducted in this study, for the primary or secondary endpoints, are for research purposes only and unless specified otherwise, do not have neither a clinically appreciable significance nor serve any diagnostic/treatment purpose.



However, if a clinical grade test result is associated with accompanying symptoms, and/or the test result requires additional diagnostic testing or medical/surgical intervention, and/or the test result is considered to be an AE/SAR by the Investigator or Sponsor it should be reported as an adverse event.

NOTE: Merely repeating an abnormal test, in the absence of any of the above conditions, does not constitute an AE/SAR. Any abnormal test result that is determined to be an error does not require reporting as an AE/SAR.

## Role of Hospitalizations in Classifying an Event

AE/SARs associated with hospitalization or prolongation of hospitalization is considered serious. Admission also includes transfer within the hospital to an acute/intensive care unit (e.g., from the cardiac wing to the medical floor for an infection, or from the medical division to the neurologic unit for a stroke).

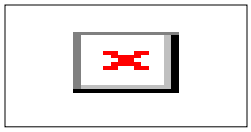
Hospitalization does not include rehabilitation facilities, hospice facilities, respite care (i.e., caregiver relief), skilled nursing facilities or homes, routine emergency room admissions, or same day surgeries (as outpatient/same day/ambulatory procedures). Hospitalization or prolongation of hospitalization in the absence of a precipitating, clinical AE/SAR is not in itself an SAE/SSAR.

## Unanticipated Problems (UPs)

An UP is an incident, experience, or outcome that specifically causes increased risk to the study or to its participants which may be of medical or non-medical etiology, and meets the following criteria:

1. Unexpected (in terms of nature, severity, or frequency), given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied
2. Definitely, probably, or possibly related to participation in the research (i.e., there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures or materials involved in the research); and
3. Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized
4. All UP reporting will follow the same guidelines as noted above for SAE/SSAR reporting, and must include a corrective action plan/measures to prevent recurrence.

## Reporting by Principal Investigator



For all events (AE/SAR and SAE/SSAR), monitoring and reporting to the PI or EC begins at the time that the participant provides informed consent, which is obtained prior to the participant's participation in the study, i.e., prior to undergoing any study related procedure and/or receiving study supplement, through and including 30 calendar days after the subject completes the study. Events should be recorded on the Adverse Event electronic case report form (eCRF). Do not delay the initial reporting of an event in order to obtain resolution or follow up information.

For all events, the PI must pursue and obtain adequate information both to determine the severity and causality of the event. For events with a causal relationship to the study supplement, follow-up by the Investigator is required until the event or its sequelae resolve or stabilize at a level acceptable to the PI, who concurs with that assessment.
In the rare event that the PI does not become aware of the occurrence of a SAE/SSAR immediately (i.e., if an outpatient study subject initially seeks treatment elsewhere), the PI is to report the event within 24 hours after learning of it and document the time of his/her first awareness of the event.

## Severity Assessment

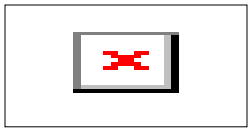
The Common Terminology Criteria for Adverse Events (CTCAE), version 5.0, for detailed descriptions of Severity Grades has been used in this study. The CTCAE schema is classified by body system and event using the Medical Dictionary for Regulatory Activities (MedDRA) System Organ Classification (SOC) hierarchy and provides descriptions of events that qualify under each severity rating. The CTCAE v5.0 schema was accessed from https://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/CTCAE_v5.0.xlsx and https://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_5x7.pdf.

The following table contains general descriptions of Adverse Event Severity Grades.

*Please note: Grade 1 (Mild) AE/SARs are not entered in the electronic CRF*.

## *CTCAE Severity Grading Scale*

| Severity Grade | Description |
|---|---|
| 1 | Mild. Asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention is not indicated. |
| 2 | Moderate. Minimal, local, or non-invasive intervention indicated or limiting activities of daily living (i.e. preparing meals, shopping for groceries/clothes, managing money, using telephone, etc.) |
| 3 | Severe or medically significant but not immediately life-threatening. Hospitalization or prolongation of hospitalization indicated OR disabling OR |



| | |
|---|---|
| | limiting self-care (e.g. bathing, dressing, feeding self, using toilet, taking medications, etc.) |
| 4 | Life-threatening consequences; urgent intervention indicated. |
| 5 | Death. Death related to adverse event. |

Notice that severity and seriousness are different concepts. For example, a headache may be severe (interferes significantly with subject's usual function) but would not be classified as serious unless it met one of the criteria for SAE/SSARs.

### Causality Assessment

The study nomenclature for assessing the causal relationship between the study supplement and an event is listed in the following table.

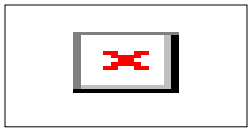
### *Adverse Event/Suspected Adverse Reaction Relationship to Study Supplement*

| | |
|---|---|
| Unrelated | No temporal association to study supplement<br>An alternate etiology has been established. |
| Unlikely | Clinical events that are likely to be caused by participant's clinical state, environment or administration of other therapies or exposure to toxins |
| Possibly Related | Reasonable temporal relationship to study supplement.<br>Connection to study product cannot be ruled out |
| Probably Related | There is a reasonable temporal association with the study supplement.<br>There is a high degree of certainty that the event is related to the study supplement. |
| Definitely Related | There is a direct temporal relationship to the study supplement.<br>The event follows a known pattern of response to the study supplement. |

The PI chooses the category that overall best describes the relationship between the event and the study supplement and records the evaluation on the Adverse Event eCRF. Note: If the PI is unable to identify whether or not the study supplement caused the event, then the event will be handled as "possibly related to investigational supplement" for reporting purposes.

## 14. DATA AND SAFETY MONITORING BOARD

The Data and Safety Monitoring Board (DSMB) will comprise of four experts responsible for reviewing clinical trial data on an ongoing basis to ensure the safety of study participants and validity and integrity of the data. Members will be independent, with no vested interest in the specific outcomes of dietary supplementation. The DSMB will review evidence of adverse events and interim outcomes to recommend whether the trial should be continued, altered, or



terminated. The DSMB will have access to “unmasked” data during the course of the trial, meaning that DSMB members know which subjects are in which supplement group.

## Study Review

The DSMB will be responsible for reviewing study documentation (e.g., study protocol, the informed consent and other participant handouts, etc.) and ensuring that it has adequate information to assess the safety of the study participants and the efficacy of the intervention during the study period. The DSMB will ensure that the data and safety monitoring plan is sufficient, given the complexity of the study and participants.

## Study Evaluation

The DSMB will evaluate participant safety data throughout the duration of the trial; evaluate the efficacy of the study intervention at intervals specified in the DSMB charter (described below); and independently provide recommendations to the study Executive committee and the IRB to either continue, amend or terminate the clinical trial based on this information. The presence of early unanticipated supplementation results, side effects, or adverse events are all reasons that a DSMB might recommend termination of the clinical trial early due to safety or efficacy matters.

## Study Monitoring

The DSMB will be responsible for monitoring the performance of clinical recruitments (e.g., protocol violations, improper participant enrollment criteria, slow accrual rate, low participation rate, failure of randomization, inadequate supplementation adherence, inadequate follow-up rate, severely compromised validity). The DSMB will independently make recommendations to the EC for improving the performance of the study or terminating the study if it determines the study would be unable to provide useful data, regardless of modifications. A summary of each board meeting and the board recommendation(s) will be provided to the EC and the IRB.

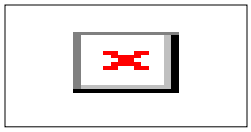
The DSMB will use a combination of, notifications, event specific reports, and scheduled cumulative trial reports to keep the EC and the IRB informed about real and potential safety issues.

## Notifications

Notifications are comprised of an email to the EC and DSMB with available information on the date and nature of the event, the study physician’s evaluation of the severity, expectedness, and relatedness to study supplement; and the assessment of the event given the information known at the time of the initial reporting.

## Event Specific Reports

Event specific reports are formal written reports providing the details of the event (including circumstances surrounding the event, laboratory testing, concomitant medications, and any formal diagnoses made via medical intervention). These reports include a full assessment by



the study physician of the severity, expectedness, and relatedness to study supplement as well as any available status update on the participants.

## Scheduled Cumulative Trial Reports

Scheduled cumulative trial reports will be prepared semi-annually by the study PI. These will be used by the DSMB to assess recruitment, subject safety, and continued trial feasibility. These reports include total numbers of AE/SARs and SAE/SSARs experienced in the overall trial. The information provided includes both new events reported since the last DSMB meeting and cumulative events reported during the life of the trial.

## CTU Reporting Requirements to DSMB and IRB

Once the event has been reported to the PI or the study physician, it will be reported to the Executive Committee within 48 hours. The UofL CTU (part of the EC) will use the Medical Dictionary for Regulatory Activities (MedDRA) System Organ Classes (SOC) to classify all AEs/SARs (including SAEs/SSARs assessed by PI or study physician). Additional supporting documentation may be requested from the PI and study team to enable the UofL CTU to accurately assess the event for reporting.

**Reportable Events**:

All unexpected events, all events related to study product, and all events with a severity grade of 3 or higher are reported by the UofL to the DSMB and the IRB.

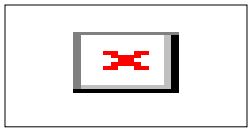
**Reporting Timeframe**:
For events which are unexpected AND associated with study supplement, the CTU will notify the IRB and DSMB within 72 hours of learning of the event; with an event specific report filed no later than 7 days post notification.

For all other reportable events, the CTU will notify the IRB and DSMB no later than 15 days of learning of the event; with an event specific report filed no later than 30 days post notification.

Cumulative trial reports (which include all events) will be generated for review by the CTU and DSMB at semi-annual DSMB meetings.

## Reporting Requirements to the FDA

Carnosine is a freely available over the counter dietary supplement in the market. This is not an experimental drug or an untested product. Commercially available Carnosine (or beta-alanine) is well known in the market and it is generally recognized as “safe when used in accordance with good manufacturing practice”. The goal of this study is to provide dietary supplementation of a naturally occurring amino acid inside the body and to study whether dietary supplementation can enhance normally occurring physiological/biochemical processes in the body.



As per the Office of Inspector General of the Department of Health and Human Services' report on "Dietary Supplement Labels: Key Elements" – "*The Dietary Supplement Health and Education Act of 1994 (DSHEA) [44] defines the term "dietary supplement" to mean a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of any of the aforementioned ingredients*". [47] As per the DSHEA, dietary supplements are not regulated by the FDA other than for their good manufacturing practices. [44]

Hence, the study (EC or DSMB) has no obligations to report any findings to the FDA. The study will however take every precaution to ensure that the supplement providers (bulk supplier and pharmacist) are operating in facilities at par with good manufacturing practices and are in possession of appropriate certifications and licensure. These documents can be found in Appendix D.

## Guidelines for Canceling Study Supplement Administration

The events listed below will follow the same reporting criteria for SAE/SSARs as it relates to the PI as well as the CTU and are to be considered sufficient to halt the study procedure. If subject develops any of the following conditions within 48 hours of planned supplement consumption, study supplement delivery will be halted in that particular participant:

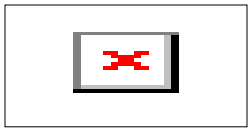
1. Diarrhea / Vomiting / Stomach Upset
2. Sudden drop in blood pressure
3. Sudden drop in blood sugar
4. Hemodynamically unstable

## Holding Rules

The expected number of SAE/SSARs in this trial is anticipated to be too small to support formal statistical stopping rules. However, the following criteria are provided as guidelines under which this **clinical trial may be put on hold pending a detailed investigation by the DSMB**. The assessment of the event shall include the relationship to the trial procedures as unrelated or possibly related. If possibly related, an indication of causality to a specific aspect of the study supplement / procedures will be determined: (e.g. study supplement consumption related, study supplement safety related, study supplement manufacturing related).

## Holding Criteria

The DSMB will recommend putting the trial on hold as soon as it is notified of **one or more of holding criteria** listed below. The study holding criteria have been created under the guidelines of current literature data and the 21 CFR code of the FDA regulatory information. The study holding criteria:



1. 20% or more cases of out of the total enrollment with Diarrhea / Vomiting / Stomach Upset
2. 20% or more cases of out of the total enrollment with Sudden drop in blood pressure
3. 20% or more cases of out of the total enrollment with Sudden drop in blood sugar
4. 20% or more cases of out of the total enrollment with Hemodynamically unstable events
5. 20% or more cases of out of the total enrollment with Hospitalizations
6. Or any other reason specified by the DSMB, study EC, bulk supplier or manufacturing pharmacist

As soon as any of the above is identified, the EC will inform the IRB and the DSMB. In addition, the DSMB will monitor the distribution of all SAE/SSARs, and the PI will be responsive to all DSMB concerns regarding SAE/SSARs that are not part of holding criteria listed above.

In addition, the DSMB may also recommend stopping the trial for the performance-related issues that would prevent the study from meeting its scientific objectives, such as:

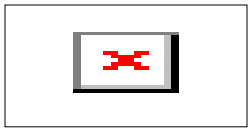
- Failure to recruit and enroll participants
- Inability to meet protocol requirements for delivery of study supplements at specified times
- Inability to prepare study supplement which meets quality specifications

# 15. STATISTICAL PROCEDURES

## Randomization

Randomization to Carnosine assignment will be conducted using a statistical software by one of the study statisticians. The randomization key detailing which participant is in which group (Carnosine or placebo) will be maintained by the Randomization Committee (RC) consisting of the study physician (Andrew DeFilippis, MD) and a member of the UofL CTU. After the investigator or research coordinator verifies that screening testing is complete and the inclusion and exclusion criteria for the study have been satisfied, an un-blinded member of the Randomization Committee will have secured access to the participant's supplement assignment.

Participants will be randomized to the Carnosine or placebo group, using a block size of 6. The stratification factors are sex, age (two group) and carnosine level (two group). When a participant is identified from the screening process (data from Visit # 1), the clinical recruitment team will be informed whether the participant will be receiving supplements from pill-box labeled "A" or "B. The clinical recruitment team will assign the participant a study ID from a pre-defined series, which will have no bearings on supplement randomization process.



As detailed in Section 11 “Study Supplement”, the University of Colorado Skaggs School of Pharmacy and Pharmaceutical Sciences will ship 28-day pill-boxes (total 3 per participant) pre-filled with either Carnosine or placebo. These boxes will also be pre-labeled with large sized visible letters “A” or “B”. The entire study team, excluding the Randomization Committee, will be blinded to whether “A” or “B” series contains Carnosine versus placebo. The University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences personnel will ensure that there is absolute consistency in the contents of each series of labeled boxes. The University of Colorado Skaggs School of Pharmacy and Pharmaceutical Sciences will directly and securely transmit the randomization key and other relevant information to the Randomization Committee (study physician and UofL CTU).

Participants of both the groups (“A” or “B”) will undergo the exact same procedures of data collection as detailed in section 10 “Study Visits”. The CTU will monitor participant recruitment by providing reports to the study co-PI and DSMB as appropriate during the recruitment phase. Updated reports will be maintained on an Internet site accessible to all units of the study. The recruitment reports will provide data on recruitment of women and minorities (African-Americans, Hispanics, and Asians). Goals for recruitment will be set and will be reviewed by the PI.

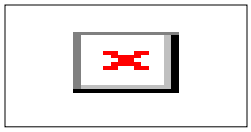
## Statistical Analyses

Biostatisticians at the Envirome Institute, with the assistance of bioinformatics experts, have adapted or developed a number of statistical programs for analyzing study data. Data are analyzed for both data monitoring purposes, as described above, and for the purpose of detecting effects of the supplement. The Envirome Institute uses standard statistical packages such as SAS, S-PLUS, R and Stata to perform statistical analyses.

Participants receiving supplementation will be presented. Participants receiving supplementation who are found not to have fully met the eligibility criteria will also be presented. On-study protocol violations will also be presented. Participants who do not complete the required observations will be listed and evaluated separately as necessary. Reasons for study discontinuation and date of withdrawal from study will be presented.

## Baseline Analyses

Although the stratified random assignment of participants to the various supplements should ensure comparability with respect to known and unknown variables, imbalance may occur by chance. Descriptive statistics for baseline characteristics known or suspected to be associated with outcomes will be prepared for the various supplementation groups. The variables considered in such a description can be categorized as: 1) demographic characteristics; 2) medical history; 3) physical examination; and 4) laboratory data. Exact testing for categorical variables and Student *t* testing for continuous variables will be used to evaluate the differences in baseline variables between supplement groups.



## Outcome Analyses

### *Primary and Secondary Endpoints*

There are 5 primary endpoints: difference in EPCs (EPC-2, EPC-3, EPC-4, EPC-7, and EPC-8) between Carnosine and placebo groups at 6 weeks and 12 weeks. These endpoints will be evaluated at the alpha=0.01. There are four secondary endpoints: difference in EPCs between Carnosine and placebo groups at 12 weeks, difference in EPCs between Carnosine and placebo groups at 6 weeks, difference in arterial stiffness between carnosine and placebo groups at 6 and 12 weeks, difference in endothelial microparticles between carnosine and placebo groups at 6 and 12 weeks, and difference in platelet monocyte aggregates between carnosine and placebo groups at 6 and 12 weeks.

Continuous variables will be presented by summary statistics (such as mean, median, standard error, range, 95% CI, correlations) and the categorical variables by frequency distributions (frequency counts, percentages and 95% CI). We will also calculate correlations along with confidence intervals. To identify significant differences due to intervention, we will compare the differences using a two sample t-test. We will use regression (logistic or linear) to model and test the effect of other covariates such as age and race. We also will compare outcome measures using GLM and MIXED model procedures to account of repeat observations. General statistical analyses will be performed with the SAS statistical software package (SAS Institute Inc., Cary, NC).

## Statistical Monitoring Rule

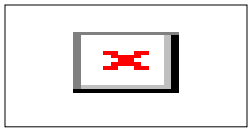
Safety monitoring of the accumulated outcomes data is designed to ensure the continuing safety of the currently enrolled participants and participants not yet enrolled. This is achieved by stopping the trial early to reduce the number of participants exposed to a harmful or ineffective supplementation.

### *Monitoring Non-efficacious Supplementation (Futility)*

We do not plan any interim analyses for futility due to longer follow-up (1 year).

### *Monitoring Toxic Supplementation (Safety)*

The cumulative number of grade 3 or 4 toxic events will be monitored after each person is enrolled [48]. If the cumulative number of toxic events produces enough evidence to conclude that the true toxicity rate is greater than or equal to 33% (Pt0 = 0.33) then the trial will be stopped early for safety reasons. If the cumulative number of toxic events after person is treated is greater than or equal to the associated boundary value bi then the combination supplementation is rejected for safety considerations (monitoring table with these boundaries will be generated using Ray and Rai, 2011)[48]. With this rule, there is only a 5% chance of stopping the trial early for lack of safety if the true toxicity rate is less than 33%. Continual assessment of the toxic events ensures we do not expose an undue number of participants to a harmful supplementation.



A second sub-analysis to be executed is the effect of Carnosine supplementation in participants with cardiovascular disease compared to the effect of Carnosine supplementation in participants without any cardiovascular disease.

## Subgroup Evaluations

The effect of subgroup stratum on the relationship between carnosine supplementation and the endpoints (both primary and secondary) will be assessed. If a carnosine supplementation effect is demonstrated, it is not likely to behave identically among all important subgroups. The subgroups of interest are age, sex, race, socioeconomic status, diabetes, smoking, hypertension, and cardiovascular disease risk scores.

These additional analyses can sometimes be helpful in identifying extreme differences in the effects of supplementation among subgroups, although the literature wisely warrants that caution be used in interpreting subgroup analyses.

## Additional Analyses

We will use the 6 week and 12-week EPC levels to assess the change of EPCs from baseline to 6 weeks and 12 weeks, assessing if this trajectory is related to carnosine supplementation using mixed model analysis of variance.

We will evaluate the relationship among the indices of the primary endpoint – EPCs. Specifically, we will assess the relationship between the differences in EPCs, adjusting for important baseline covariates.

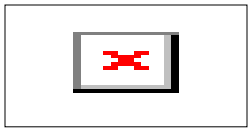
## Multiple Comparisons

In this study, no adjustments have been made for multiple comparisons. Measures of effect will be effect size, the standard error of the effect size, the 95% confidence interval for the effect size and the *p*-value. *P*-values will be interpreted at nominal 0.05 levels.

# 16. TRIAL MANAGEMENT

## Institutional Review Board (IRB) AND Informed Consent

This protocol and the informed consent document and any subsequent modifications will be reviewed and approved by the IRB or ethics committee responsible for oversight of the study. A signed consent form will be obtained from the participant. The consent form will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. A copy of the consent form will be given to the subject and this fact will be documented in the participant's medical record if recruited from a clinic.



## Informed Consent Process

Potential participants will be approached by one of the study investigators or research coordinators. Information regarding study participation will be provided to the potential participant. The informed consent includes descriptions of all study related procedures, all possible risks to participant, and the time commitment involved with participating. All consent forms will have IRB approval. Individuals who agree to participate will receive a copy of the signed informed consent. The research staff member obtaining consent will document the informed process in the participant's chart if recruited from a clinic.

## Risks Associated with Manufacturing and Consuming of Dietary Supplement

### *Manufacturing Dietary Supplement*

Supplement manufacturing is done under strict sterile conditions; however, there is a rare chance that the capsules could become contaminated while being processed. Quality control checks will be done on the manufacturing process, and if the tests reveal break in protocol or contamination, the participant will be notified and instructed on whether or not he/she should be treated with antibiotics. The participant will be diligently assessed for adverse reactions or events at each follow-up visit (Visit # 3 and Visit # 4). If the participant notes a fever, he/she will be requested to notify the investigator/study team immediately.

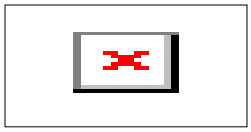
### *Consuming Dietary Supplement*

Possible risks of Carnosine supplementation include: tingling of upper / lower extremities, drop in blood pressure, nausea, vomiting, stomach upset.

Participants who are taking antihypertension medications at the time of the study may be susceptible to a temporary sudden drop in blood pressure; during which time the participant may be at an increased risk of stroke and heart attack. The participant will be advised to inform the research team immediately of any symptoms of dizziness, light-headedness, chest pain, chest tightening, nausea, blurred vision, slurred speech, facial drooping, decrease sensations anywhere on your body, or weakness or a decrease in strength of the arms or legs. The participant will be closely monitored if started back on the study supplement regimen. The study physician will conduct a detailed assessment and provide its recommendations to the PI.

## Adequacy of Protection Against Risks

The precautionary measures mentioned in the last two sections will minimize the risk associated with taking study dietary supplement. Overall the study procedures are low risk and in previous similar trials there was no complications related to Carnosine or placebo supplementation in healthy participants.[34, 37]



## Potential Benefits of the Proposed Research to the Participants and Others

The potential benefits of this research for participants include reduced oxidative stress from $PM_{2.5}$ pollution which will have therapeutic effects such as decrease in CVD progression and ultimately decreased CVD mortality. This project will also provide mechanistic insight into Carnosine supplementation which will be useful for finding new treatments for other diseases.

## Risk Benefit Analysis

The supplementation of dietary Carnosine offers a new option to participants with existing CVD of different intensities. The goal of this supplementation is to improve ameliorate the progressive nature of CVD by facilitating the reduction of oxidative stress on vasculature. Having highly trained experts oversee the supplementation with close study monitoring substantially reduces the likelihood of AEs. The potential risks to the participants remain reasonably low in relation to the possible benefit of improving their CVD risk factors during high pollution season, without the involvement of prescription medications or invasive diagnostic / treatment procedures.

## Importance of Knowledge to be Gained

The knowledge to be gained from this clinical trial is significant in that 1) participants with high exposure to $PM_{2.5}$ environmental pollution and related CVD risk factors may benefit from receiving a freely available over the counter dietary supplement that heretofore has demonstrated few risks to the participant.

The study has been designed to address critical limitations in the previous published studies which have not looked at the effect of Carnosine supplementation on mitigating CVD risk factors. The risks to the participants are reasonable in relation to the knowledge gained from this study since this supplementation therapy may potentially reduce the widely prevalent and debilitating effects of $PM_{2.5}$ pollution.

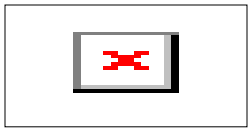
## Data Safety Monitoring Board (DSMB)

The Data and Safety Monitoring Plan has been outlined in Section 16 above.

## Clinical Recruitment

The PI and other involved investigators will oversee that the clinical research team and investigators are trained on but not limited to the following topics:

1. Maintaining IRB required trainings and certifications (e.g. CITI program, etc.)
2. Obtaining informed consent
3. High quality data collection
4. Good clinical practice
5. HIPAA privacy
6. Institutional compliance
7. Understanding the nature of the protocol or investigational plan.



8. The study PI understands and accepts his or her obligation to obtain IRB review and approval of a clinical investigation before the investigation may be initiated and to ensure continuing review of the study by the IRB in accordance with 21 CFR Part 56, and to keep the sponsor informed of such IRB approval and subsequent IRB actions concerning the study.
9. Access to an adequate number of suitable potential candidates / participants to conduct the investigation.
10. Adequate facilities for supplement preparation and conducting the clinical investigation.
11. Sufficient time to carry out the responsibilities of best practices of clinical research
12. The PI understands periodic / random audits by the IRB may occur

## Database

The Envirome Institute will maintain a study database in a web-accessible electronic format. Detailed documentation of study variables will be prepared and available to study Investigators, and where necessary, to external scientists. Appropriate confidentiality and security of these files will be maintained at all times.

## Security

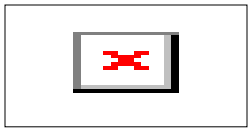
Several levels of security will be implemented to protect the confidentiality of the data. All authorized users will be provided a unique name/password and will be given access as identified by the Principal Investigator. Data will be stored on a REDCap database which is HIPAA compliant database routinely utilized by clinical scientists nationwide to store sensitive research data.

## Data Quality

All data will have to pass through range and logical checks in addition to intra- and inter-form checks for consistency. The sequence of events will be enforced by allowing subordinate forms to become accessible only after its primary form has been submitted. If a response to a question on a form requires ancillary forms to be completed, the user will receive reminder messages within the application to complete the proper form. Regular reports on the REDCap data entry and completeness will be generated. Data quality or management questions or concerns will be immediately addressed.

## Dissemination

The overall usefulness of scientific research depends not only on the importance of the findings, but also on its eventual reach and effect on population health. Therefore, research projects must integrate ways to promote the eventual diffusion of the results into their research plans. The Envirome Institute will work with professional associations like the NIH, NIEHS, American Heart Association for a number of initiatives including cardiovascular diseases and environmental pollution. The Envirome Institute will use multiple general dissemination methods that will be



tailored for the target audiences (e.g. manuscripts, presentations, television, multimedia, social media).

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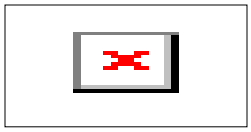

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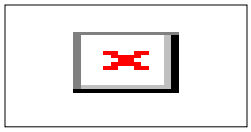

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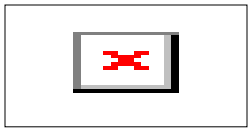

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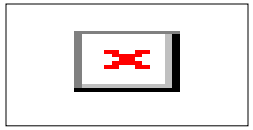


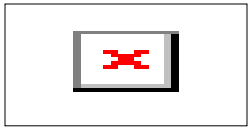
# 18. TABLES

## TABLE 1: Screening and Enrollment Timeline of NEAT Clinical Trial for Year # 1 (2020)

| Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep |
|---|---|---|---|---|---|---|---|---|
| Potential Candidates Identification | | | | | | | | |
| | Clinical Screening (Visit # 1) | | | | | | | |
| | Select Eligible Candidates from Visit # 1 | | | | | | | |
| | | | | Supplement Intervention Period (Total 12 weeks per Participant) | | | | |
| | | | | Baseline Assessment (Visit #2) Start Supplement | | | | |
| | | | | | Follow-Up # 1 at 6 weeks (Visit # 3) | | | |
| | | | | | | Follow-Up # 2 at 12 weeks (Visit # 4) | | |

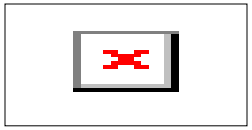
## TABLE 2: Screening and Enrollment Timeline of NEAT Clinical Trial for Year # 2 (2021)

| Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep |
|---|---|---|---|---|---|---|---|---|
| Potential Candidates Identification | | | | | | | | |
| | Clinical Screening (Visit # 1) | | | | | | | |
| | Select Eligible Candidates from Visit # 1 | | | | | | | |
| | | | | Supplement Intervention Period (Total 12 weeks per Participant) | | | | |
| | | | | Baseline Assessment (Visit #2) Start Supplement | | | | |
| | | | | | Follow-Up # 1 at 6 weeks (Visit # 3) | | | |
| | | | | | | Follow-Up # 2 at 12 weeks (Visit # 4) | | |



## Table 3: Schedule of Procedures At Each Visit / Encounter

| Study Activities | Visit # 1 (Screening) | Visit # 2 (Baseline Assessment) | Visit # 3 (Follow-Up # 1) | Visit # 4 (Follow-Up # 2) | 4-12 Month Safety Check |
|---|---|---|---|---|---|
| Informed Consent | X | | | | |
| Contact & Demographics | X | X | X | X | |
| Medical History | | X | X | X | X |
| Sleep / Dietary / Social / Exposure Questionnaires | | X | X | X | |
| Blood & Urine Samples | X (Urine only) | X | X | X | |
| Physical Exam | | X | X | X | |
| Arterial Stiffness Exam | | X | X | X | |
| Physical Function Exam | | X | X | X | |
| Supplement Distribution | | X | X | | |
| Checkout (Payment) | X ($25) | X ($50) | X ($75) | X ($100) | |
| Adverse Events Check | | | X | X | X |

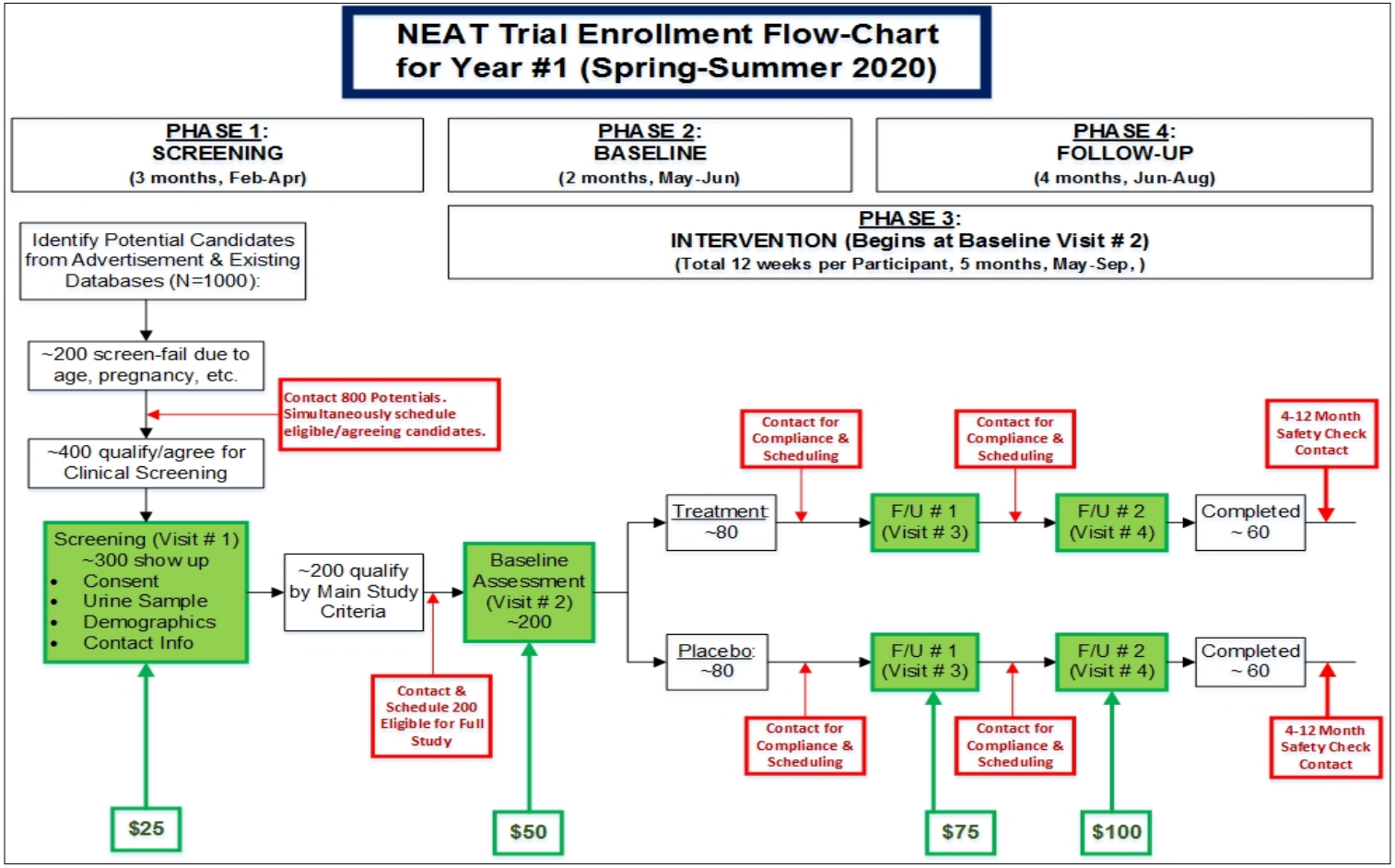


## Table 4: Study Visit Components' Time Requirements

| Study Activities | Visit # 1 (Screening) | Visit # 2 (Baseline Assessment) | Visit # 3 (Follow-Up # 1) | Visit # 4 (Follow-Up # 2) | 4-12 Month Safety Check |
|---|---|---|---|---|---|
| Informed Consent | 30 mins | | | | |
| Contact & Demographics | 5 mins | 30 mins | 15 mins | 15 mins | |
| Medical History | | | | | 2 mins |
| Sleep / Dietary / Social / Exposure Questionnaires | | | | | |
| Blood & Urine Samples | 10 min (Urine only) | 15 mins | 15 mins | 15 mins | |
| Physical Exam | | 10 mins | 5 mins | 5 mins | |
| Arterial Stiffness Exam | | 20 mins | 20 mins | 20 mins | |
| Physical Function Exam | | 20 mins | 20 mins | 20 mins | |
| Supplement Distribution | | 10 mins | 10 mins | | |
| Checkout | 10 mins | 10 mins | 10 mins | 10 mins | |
| Adverse Events Check | | | 5 mins | 5 mins | 2 mins |
| Estimated Visit Time | 60 mins | 120 mins | 100 mins | 100 mins | 5 mins |

# 19. FIGURES

## Figure 1: NEAT Study Flow-Chart for Year # 1 (2020)



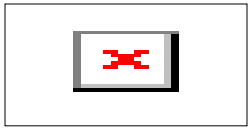


**Figure 2: NEAT Study Flow-Chart for Year # 2 (2021)**

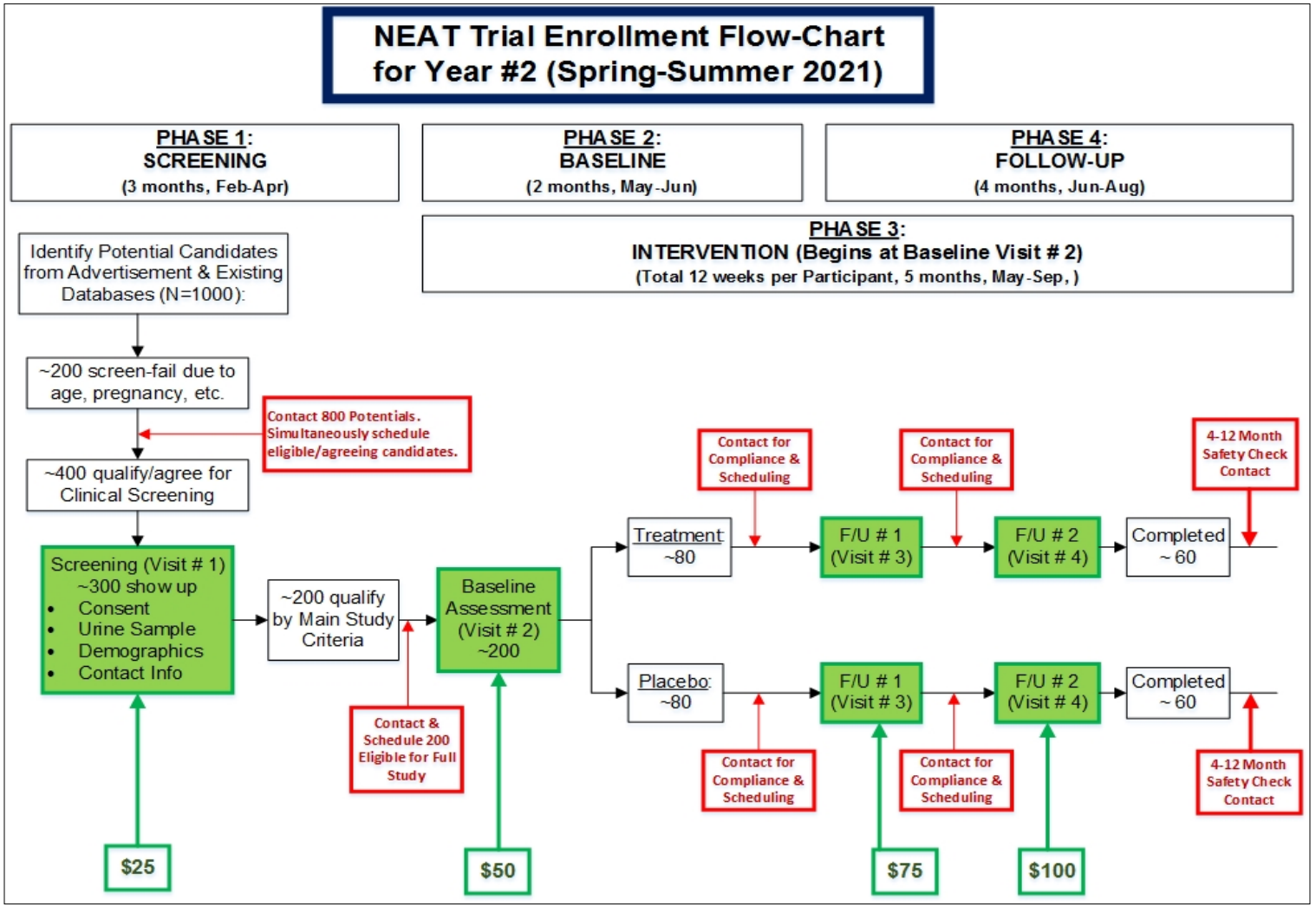


**Figure 3: Individual Visit Flow-Chart – Screening (Visit # 1)**

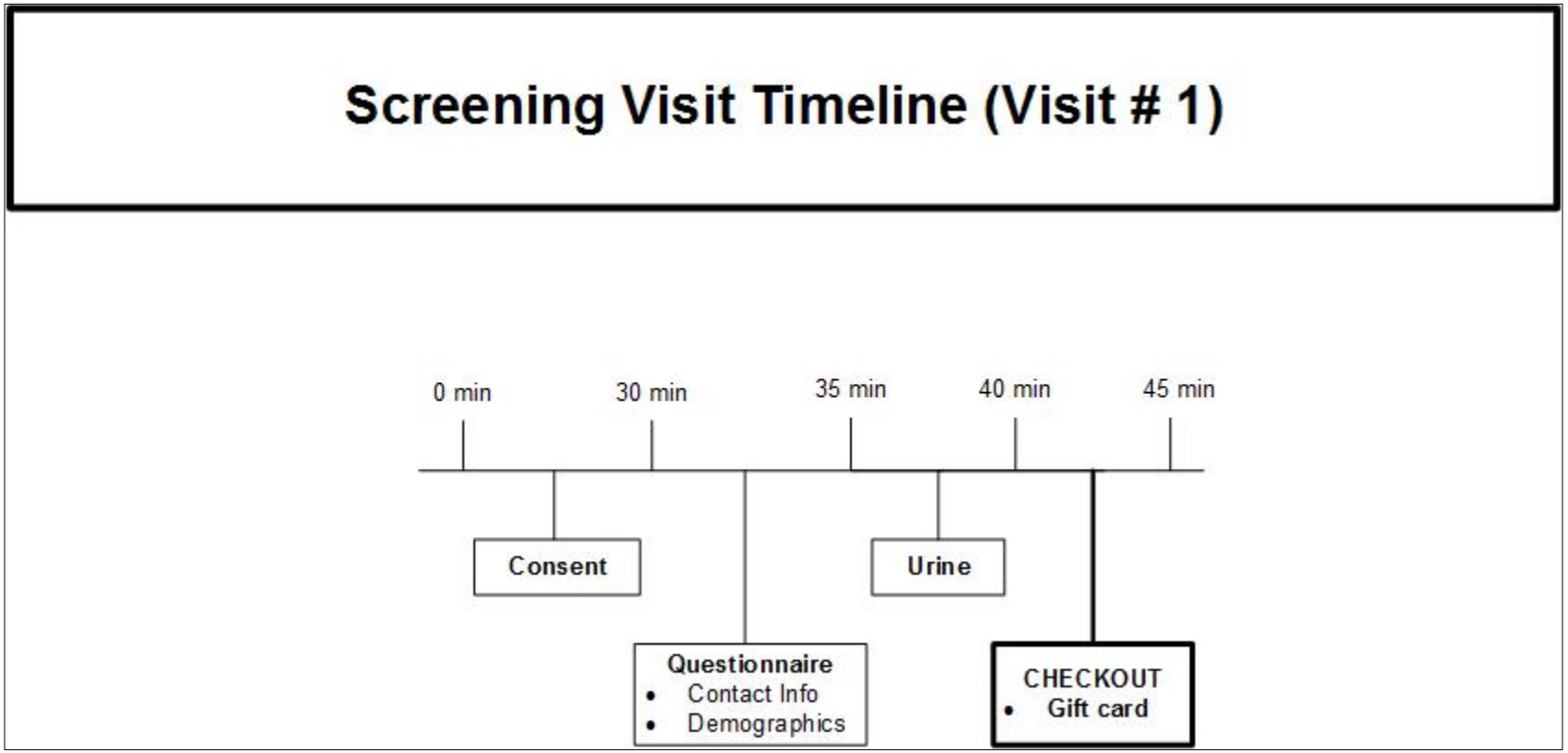


**Figure 4: Individual Visit Flow-Chart – Baseline Assessment (Visit # 2)**

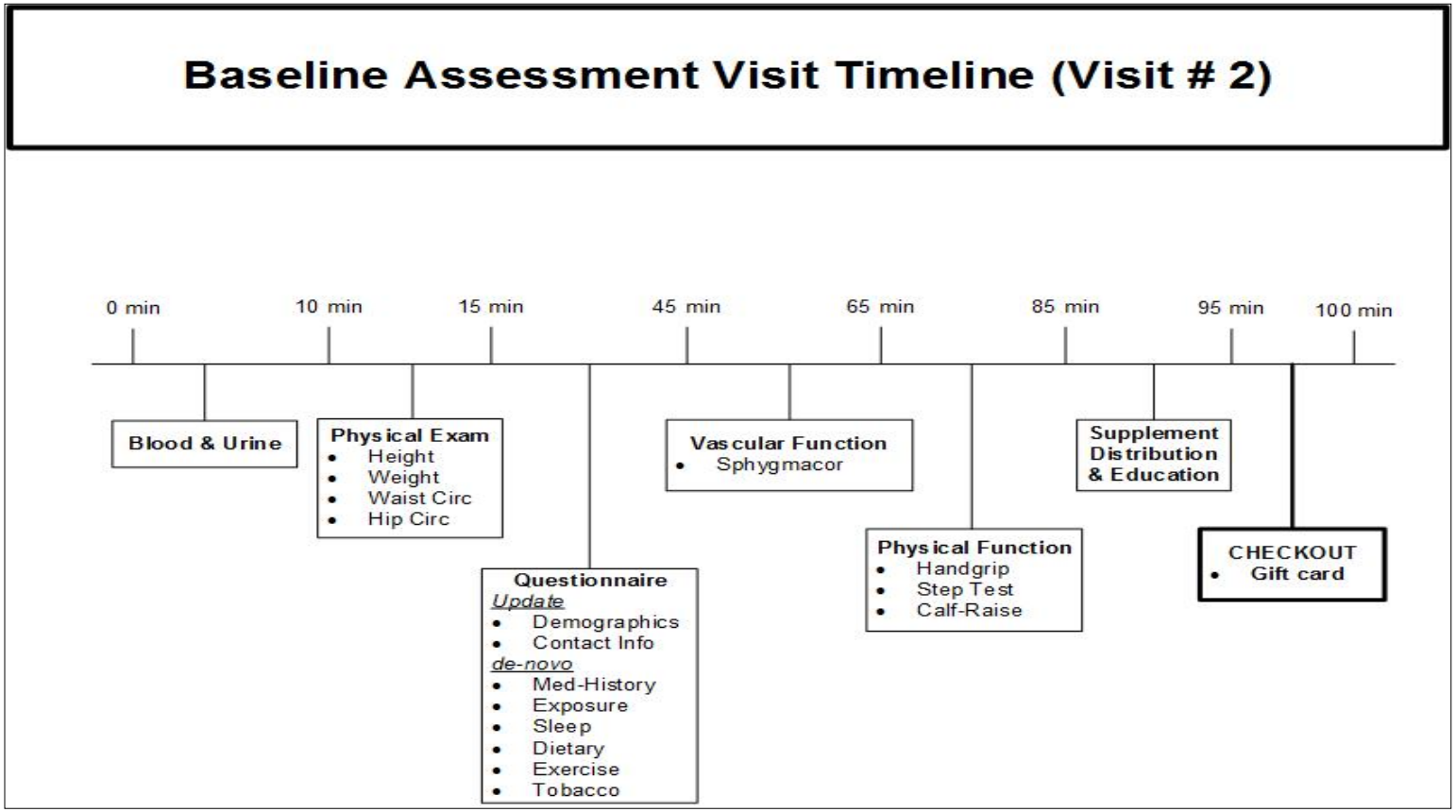


**Figure 5: Individual Visit Flow-Chart – Follow-Up # 1 (Visit # 3)**

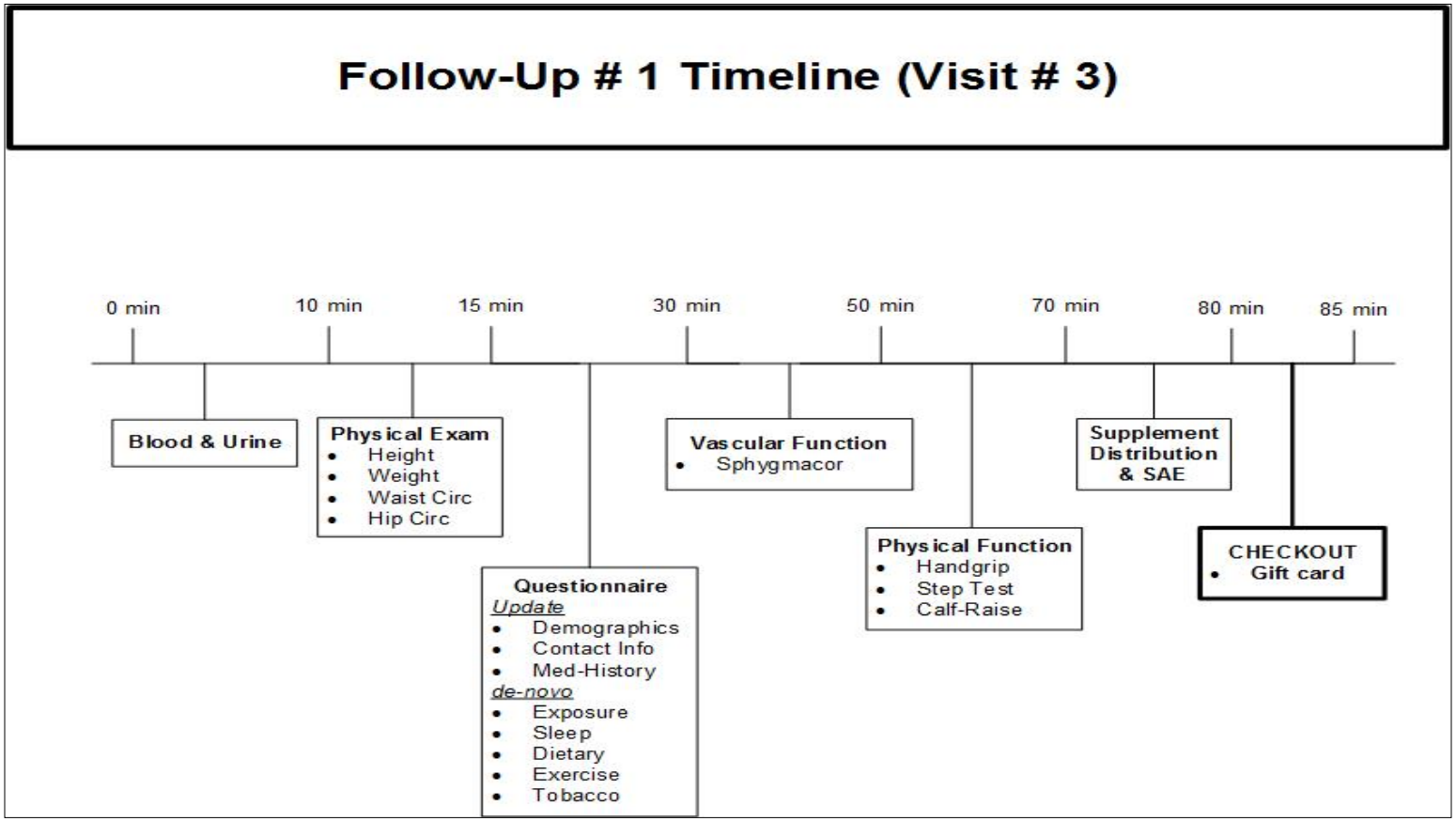
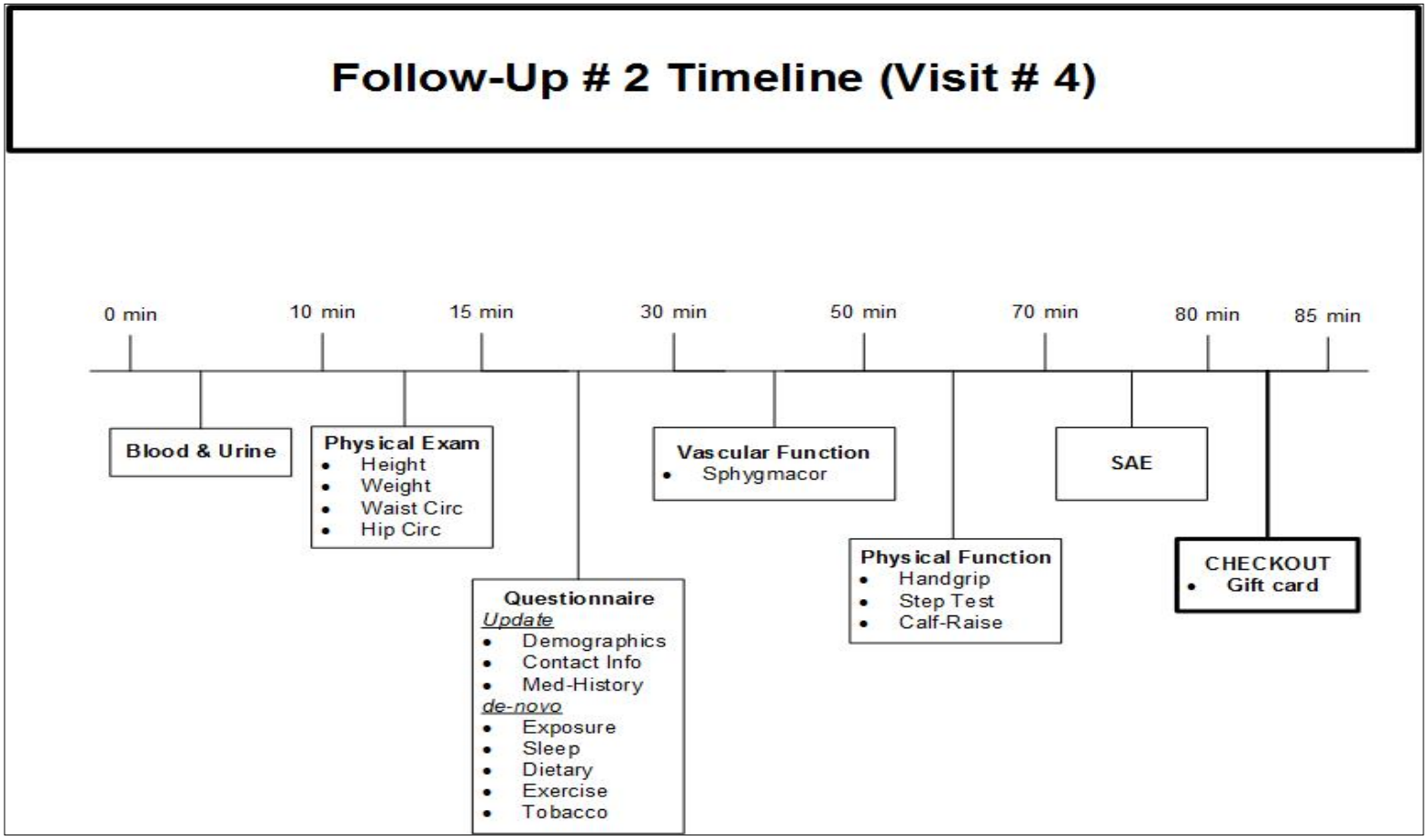


**Figure 6: Individual Visit Flow-Chart – Follow-Up # 1 (Visit # 4)**



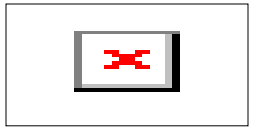
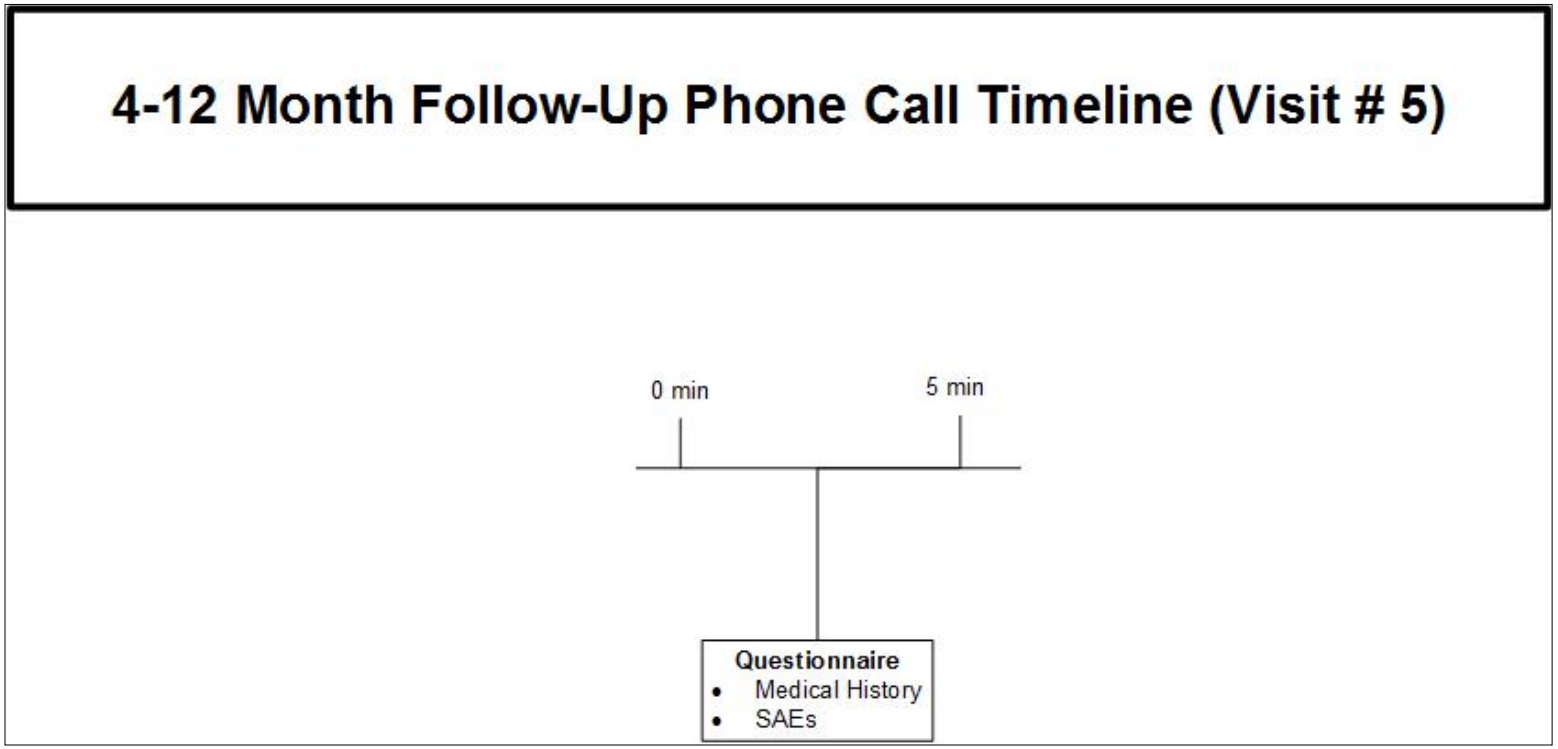
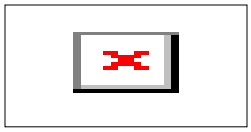


**Figure 7: Individual Visit Flow-Chart – 4-12 Month Safety Check (Visit # 5)**





# 20. APPENDIX A – SAMPLE PROCUREMENT AND PROCESSING

## Blood Sample Collection

Every effort must be made to make venipuncture as easy and painless as possible for participants. The technician must remain calm and project an attitude of competence even when faced with the most nervous or inquiring participant. The best way to achieve this is for the technician to be thoroughly knowledgeable about all aspects of the procedures. This study involves the collection of a total of ~45 ml of blood. Any participant who is concerned about the volume of blood should be reassured that the total amount of blood drawn is about 1.5 ounces, although it may look like more.

## Blood Collection Tray and Tubes *Preparation*

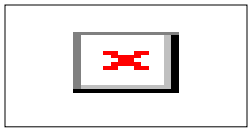
Prior to venipuncture the tray is prepared for each participant. Each tray holds the Vacutainer tubes used in blood collection labeled with the subject identifier code (given by the research coordinator), date, type of blood being collected (serum whole blood, EDTA plasma, sodium citrate plasma, etc.) and the study visit (Visit # 1, 2, 3, 4).

## Blood Collection Procedure

### *General Guidelines*

Venipuncture should be performed using standard sterile technique in the sitting position. Handle all specimens as potentially infectious for laboratory workers. OSHA rules mandate that technicians must always wear disposable protective gloves when collecting and processing specimens. All needles and sharps must be discarded into puncture resistant containers. Avoid formation of potentially infectious aerosols when removing the rubber stoppers from Vacutainer tubes. In addition to wearing protective gloves, hold a piece of gauze over the stopper while slowly removing it from the tube. Place all used Vacutainer tubes and blood-contaminated products in biohazard bags for proper disposal. Use 0.5% sodium hypochlorite (household bleach diluted 1: 1 0) to clean up any spills of blood, plasma, or serum.

**For Extremely Apprehensive Participants**: Do not under any circumstances force the participant to have blood drawn. It may help to explain to the subject that the blood drawing is designed to be nearly painless as possible. It may also be helpful to have the participant relax in the blood drawing chair just so the phlebotomist can check the veins in the participant's arms, without actually drawing blood. If the participant is very anxious, he/she may lie down during the blood draw. Give the participant enough time to feel comfortable after the blood collection, as well. In many cases the most memorable part of the experience for participants will be the contact with the technicians who draw the blood and their general attitude and competence.



## *Procedure*

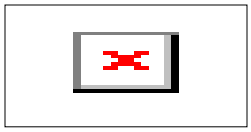
Before applying the tourniquet, screw the Luer adapter into the plastic Vacutainer tube guide. Insert the butterfly tubing onto the adapter. With jacket or sweater removed, have the participant sit upright with the sleeves rolled up to expose the antecubital fossa.
Wrap the tourniquet around the arm 3 to 4 inches (7.5 to 10.0 cm) above the venipuncture site. If the participant has a skin problem, put the tourniquet over the participant's shirt or use a piece of gauze or paper tissue so as not to pinch the skin. The tourniquet should be on the arm for the shortest time possible. Never leave the tourniquet on for longer than two (2) minutes. To do so may result in hemo-concentration or a variation in blood test values. If a tourniquet must be applied for preliminary vein selection, and it remains of the arm for longer than two minutes, it should be released and reapplied after a wait of two minutes.

**Venipuncture**:

1. Identify the vein, and then cleanse the venipuncture site.
2. Remove the alcohol prep from its sterile package.
3. Cleanse the vein site with the alcohol prep using a circular motion from the center to the periphery.
4. Allow the area to dry to prevent possible hemolysis of the specimen and a burning sensation to the subject when the venipuncture is performed.
5. If venipuncture becomes difficult, the vein may need to be touched again with your hand. If this happens, cleanse the site again with alcohol.

**After successful venipuncture**:

1. Place tubes into the Vacutainer holder.
2. Grasp the participant's arm firmly, using your thumb to draw the skin taut. This anchors the vein. The thumb should be 1 or 2 inches below the venipuncture site.
3. With the needle bevel upward, enter the vein in a smooth continuous motion.
4. Make sure the participant's arm is in a flat or downward position while maintaining the tube below the site when the needle is in the vein. It may be helpful to have the participant make a fist with the opposite hand and place it under the elbow for support.
5. Grasp the flange of the needle holder and push the tube forward until the butt end of the needle punctures the stopper, exposing the full lumen of the needle. The tube should begin filling with blood.
6. Remove the tourniquet after tube #1 fills.
7. Keep a constant, slight forward pressure (in the direction of the adapter) on the end of the tube. This prevents release of the shutoff valve and stopping of blood flow.
8. Fill each Vacutainer tube as completely as possible; i.e. until the vacuum is exhausted and blood flow ceases. If a Vacutainer tube fills only partially, remove the tube and attach another without removing the needle from the vein.
9. When the blood flow into the collection tube ceases, remove the tube from the holder. The shutoff value covers the point, stopping blood flow until the next tube is inserted (if necessary).



The tubes should be gently inverted 6-8 times immediately following removal of the tube from the adapter.

If a blood sample is not forthcoming, the following **manipulations** may be helpful.

1. Tum needle slightly or lift the holder in an effort to move the bevel away from the wall of the vein.
2. Move needle slightly in hope of entering vein. Do not probe. If not successful, release tourniquet and remove needle. A second attempt can be made on either arm.
3. Loosen the tourniquet. It may have been applied too tightly, thereby stopping the blood flow. Reapply the tourniquet loosely. If the tourniquet is a Velcro type, quickly release and press back together. Be sure, however, that the tourniquet remains on for no longer than two minutes at a time.

**At the conclusion of the draw**

1. To remove the needle, lightly place clean gauze over venipuncture site.
2. Remove the needle quickly and immediately apply pressure to the site with a gauze pad.
3. Discard needle and its cap into needle box (DO NOT ATTEMPT TO RECAP NEEDLES!)
4. Have the participant hold the gauze pad firmly for one to two minutes to prevent a hematoma.

**Bandaging the arm under normal conditions**:

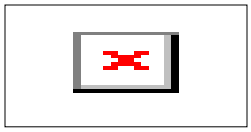
1. Slip the gauze pad down over the site
2. Continue mild pressure.
3. Apply an adhesive or gauze bandage over the venipuncture site
4. Make sure that blood flow has stopped.

**Bandaging the arm if the participant continues to bleed**:

1. Apply pressure to the site with a gauze pad.
2. Keep the arm elevated until the bleeding stops.
3. Wrap a gauze bandage tightly around the arm over the pad.
4. Tell the participant to leave the bandage on for at least 15 minutes.

**Blood Collection Precautions when a subject feels faint or looks faint following the blood draw**:

1. Have the person remain in the chair. If necessary, have him/her lie on the floor with their legs elevated. Use of a transfer belt may be indicated in this situation.
2. Take an ampule of smelling salts, crush it, and wave it under the person's nose for a few seconds.
3. Provide the person with a basin if he/she feels nauseous.
4. Have the person stay seated or lying down until the color returns and he/she feels better.
5. Have someone stay with the person to prevent them from falling and falling and injuring themselves if they should faint.



6. Place a cold wet cloth on the back of the person's neck or on their forehead.
7. Once the episode has passed, some fruit juice may be given to the participant in order to counteract any possible hypoglycemia due to fasting.
8. If the person continues to feel sick, take a blood pressure and pulse reading. Contact a medical staff member, who will advise you on further action.

## Blood Tube Labeling and Processing

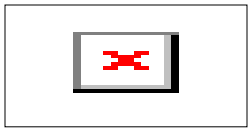
For the study, a total of approximately 45mL blood will be drawn into different tubes with or without internal coating of anticoagulants. Biomedical analysis of genetics will be performed only if the subject has consented to the genetic sub-study. It is important that the technician know more than just the arrangement of the blood collection tubes and the sequence of the tube collection. He/she should also be familiar with the purpose of each tube, the type of anticoagulant in-each tube, and possible sources of error in the handling of each tube.

Each tube should be labeled with the subject's ID code (given by the study coordinator), study ID, the date, tube type, and time point (visit # 1, 2, 3, 4)

After blood draw, all labeled tubes should be kept in a cooler until further processing and storage.

## Urine Collection

Standard clean catch urine sample will be obtained in sterile cups from participants. The samples will be transported on ice to the Diabetes and Obesity Center's research laboratory on the 4th floor of the Delia Baxter building for storage and mass spectrometric analysis. Urine samples not being processed immediately maybe placed in a biospecimens only refrigerator.



# 21. APPENDIX B – MEASUREMENT OF BLOOD BIOMARKERS

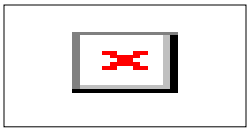
## Endothelial Progenitor Cells (EPCs)

Eight ml of venous blood collected in a CPT Mononuclear Cell preparation tube (Becton Dickinson) will be processed within 3 hours of collection. Mononuclear cells will be separated by centrifugation at 1700g for 30 minutes. The buffy coat layer will be collected and washed twice with PBS+1%BSA. Cells will be pelleted by centrifugation at 400g for 5min and incubated with Fc Block for 10 minutes (Miltenyi Biotec). 50ul aliquots will be then incubated with FITC anti-CD41a (BD Biosciences), FITC-CD235a (eBioscience), FITC-CD16 (eBioscience), QDot655anti-CD14 (Invitrogen), PE-CF594 anti-CD34 (BD Biosciences), APC anti- AC133 (Miltenyi Biotec), V450 anti-CD31 (BD Biosciences), V500 anti-CD45 (BD Biosciences), In addition, we will include a discriminator of cell viability (Live Dead Green; Becton Dickinson). An unstained cell aliquot will be used as a control. After a 30 min incubation, the cells will be washed, resuspended in PBS+1%BSA, and 50ul of volumetric counting beads (Accucount Particles; Spherotech) added. The samples will be then run on an LSR II flow cytometer (Becton Dickinson) set to acquire 20,000 beads. Collected events will be analyzed using FlowJo software. Initial gating will be selected a lymphocyte population that was negative for CD235a, CD41a, CD16, CD14 and the viability marker. Fifteen distinct EPC subgroups will be then identified by the variable expression of CD31, CD45, CD34, and AC133 (S1 Table). Positive boundaries for gating will be accomplished with the unstained sample. EPCs numbers will be quantified and normalized to volume of blood using bead counts.

## Microparticles and Platelet-Monocyte Aggregates (PMA)

For the analysis of microparticles and immune cell populations, 8 ml of blood will be collected in sodium citrate-containing cell preparation tubes (CPT Vacutainer; Becton Dickinson). These tubes will be centrifuged at 1700xg for 30 min at room temperature and shipped overnight to the University of Louisville for analysis. Upon arrival, the tubes will be centrifuged again and the upper layer containing mononuclear cells and plasma was collected. This material will be diluted with an equal volume of PBS and centrifuged at 500xg for 10 min. Aliquots of the supernatant will be used for analysis of microparticles while the cell pellet will be washed once with PBS and centrifuged. The final cell pellet will be resuspended in a volume of 300 µl PBS+2% FCS, 150 µl of which will be used for analysis of immune cell populations.

For analysis of platelet-monocyte aggregates, 3ml of blood will be collected in an acid-citrate dextrose tube (ACD Vacutainer; Becton-Dickinson), and then 1ml aliquots will be diluted with 3ml PBS and fixed with 1.3ml of 4% paraformaldehyde for 30 min on ice. Red blood cells will be lysed by addition of 24 ml of water, the samples will be centrifuged at 400xg for 10min and the cell pellet will be resuspended in 1 ml Tyrode's buffer and shipped to University of Louisville as above.



# 22. APPENDIX C – PRESCREENING COMMUNICATION SCRIPTS

## Phone Communication Script

Hi _____ (insert name), how are you today? My name is _____ (insert name) from the Department of Medicine at the University of Louisville.

The reason I am calling is to invite you to take part in a research study being conducted by Dr. Tim O'Toole. You are being invited because you are between 22-65 years of age and live in or around Louisville.

The purpose of this study is to see whether taking an over the counter carnosine supplement can help protect you from harmful effects of PM pollution, which is significantly high in the Louisville area. Carnosine is a naturally occurring chemical in our body and is easily available as an over the counter supplement, just like a protein powder! Our laboratory studies have shown that taking supplemental carnosine is protects against particulate matter pollution.

Your participation in the study would be completely voluntary.

There will be total 4 visits for the study. The first visit will tell us whether you qualify for the study. First visit will last about 45 minutes. If you qualify, we will invite you to participate in the main study, during which you will be required to take a daily dietary supplement for 3 months. You will be given either carnosine or placebo. Placebo is plain cornstarch and harmless.

There will be 3 study visits during these 3 months. All visits will be at the University of Louisville Physicians Outpatient Center (401 E. Chestnut Street 4th floor suite 460, Louisville, KY). Each visit will take about 1.5 to 2 hours and we will collect some blood, urine, and other data about your health and personal history.

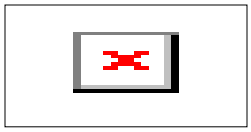
We will not do any drug testing on your samples. You will not need to answer any questions that you are uncomfortable with. If you choose to participate, as a token of appreciation for completing the study you will receive a prepaid Gift Card at each visit as per following:
$25 for Visit # 1, $50 for Visit # 2, $75 for Visit # 3, and $100 for Visit # 4.

Do you have any questions about the study?

Is this something you would be interested in doing?

(If YES, proceed. If NO, thank them for their time and discontinue the call)

If you decide to participate in the study, what would be a good date and time for you to come for a screening visit? _____ (Note down date/time for screening



visit), so that we can schedule that appointment now and we will be sending you directions for the study visit and where to come for the visit. Would you prefer to get these by mail or email? Can I verify your contact information one more time? _____(Insert contact information).

Thank you for your time and we look forward to seeing you on _____ at _____am/pm.

## Email or Letter Communication Script

Dear [Mr. / Ms. LAST NAME],

I am writing to tell you about the Nucleophilic Defense Against PM Toxicity (NEAT) study being conducted by the University Of Louisville Department Of Medicine.

We are contacting individuals who have expressed an interest in participating in research studies and live in or around Louisville. Based on your current physical address and age, we would like to invite you to learn more about the study and see if you would be interested in participating.

The purpose of this study is to see if taking a carnosine supplement can help protect against cardiovascular injury caused by exposure to naturally occurring particulate matter (PM) pollution in the Louisville area. Carnosine is a naturally occurring chemical in our body and is easily available as an over the counter supplement (just like a protein powder!).

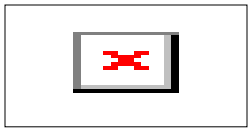
We want to learn whether taking an over the counter carnosine supplement can help protect you from harmful effects of PM pollution, which is significantly high in the Louisville area.

Your participation in the study would be completely voluntary.

A screening visit will be conducted to check whether you qualify for the main study. The screening visit will last about 45 minutes and will get some urine sample and basic information about you. If you qualify, we will invite you to come back for a visit on a later date.

In the main study, you will be required to take a daily dietary supplement of carnosine or placebo (looks like the supplement but is not) for a total of 3 months. We will see you three times during the main study period. All visits will be at the University of Louisville Physicians Outpatient Center (401 E. Chestnut Street 4th floor suite 460, Louisville, KY). Each visit will take about 1.5 to 2 hours. At each visit we will get some blood and urine samples, measure arterial stiffness using a blood pressure-like cuff, perform tests of physical strength and ask you questions about demographics, health history, and personal habits.

We will not do any drug testing on your samples. You will not need to answer any



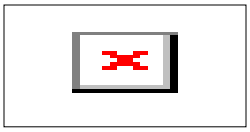
questions that you are uncomfortable with. If you choose to participate, as a token of appreciation for completing the study you will receive a prepaid Gift Card at each visit as per following:
$25 for Visit # 1, $50 for Visit # 2, $75 for Visit # 3, and $100 for Visit # 4.

Your participation in this study is voluntary.

If you are interested in learning more about this study we would be excited to speak with you and give you more details if necessary, please call us at 502-852-7559 or email us back at Envirome@Louisville.edu.

Thank you for your time and consideration. We look forward to hearing from you.
Sincerely,

Timothy O'Toole, PhD
Assistant Professor, University of Louisville, Department of Medicine



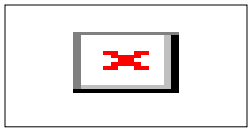
# 23. APPENDIX D – SUPPLEMENT MANUFACTURING AND QUALITY CONTROL PROTOCOL

## L-Carnosine Powder Certificate of Analysis

The certificate of analysis for the L-Carnosine powder from www.bulksupplements.com is attached. It highlights that the dietary supplement conforms the regulatory standards.

## Supplement Compounding and Quality Control Standard Operating Protocol

The capsule sources, compounding methods, quality control measures and other standard operating protocol are detailed in the attached agreement letter from the University of Colorado Skaggs School of Pharmacy and Pharmaceutical Sciences.



# 24. APPENDIX E – PHYSICAL FUNCTION MEASUREMENTS

## Handgrip Strength Test

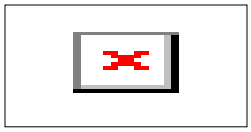
The first is the handgrip strength test, which assesses upper extremity strength and is reported to be a reliable indicator of a variety of impaired cardiopulmonary conditions. [49, 50] The handgrip strength static test will be assessed as described [51] using a Jamar Technologies Plus+ Digital Hand Dynamometer (JLW Instruments, Chicago, IL, USA). Subjects will be seated in an upright position with their shoulder adducted and elbow flexed at 90°, with the forearm allowed to rest lightly on the arm of the chair or on the subject's thigh. For each subject, a maximal voluntary contraction will be determined as the highest of three initial contractions (INITIAL MAX). [52] After a one-minute rest period subjects will be instructed to squeeze the dynamometer in a static contraction at a level of 50% of their maximal voluntary contraction for as long as possible, and the duration recorded (DURATION). [53] The level of static contraction will be monitored by the assessor who provides verbal feedback to ensure that a level of 50% maximal voluntary contraction is maintained. Subjects will then perform an additional post-fatigue maximal contraction within 10 seconds of completion of the 50% maximal static contraction (FINAL MAX). Handgrip testing will be performed on both the dominant and non-dominant hands for each subject. In addition to (INITIAL MAX), (FINAL MAX) and (DURATION), additional calculated indices will be: a) work performed (WORK = DURATION x 50% INITIAL MAX); and b) Strength Decrement Index (SDI = 100 x (INITIAL MAX + FINAL MAX)/INITIAL MAX).

## Two Minute Step Test (2MST)

The 2MST is an assessment of aerobic endurance and can be predictive of morbidity and mobility in older adults or those with CVD. Higher scores on the 2MST indicate increased functionality, quality of life, and decreased incidence of chronic health problems. [54] To perform the test, participants are asked to stand next to a wall and march in place with the intent of raising the knees to a marked target on the wall mid-way, representing a height between the lateral condyle and anterior superior iliac spine of the participant. The test administrator counts the number of times the right knee reaches the marked target over a 2-minute period. If an individual cannot march for the full 2-minutes, they are permitted to take a standing or seated rest break and resume within the 2 minutes. [55]

## Bilateral Calf-Raise Test

This is a muscle strength and endurance test that has been used to evaluate the functional performance of older adults and those with CVD.[56-59] Initially, participants will stand barefoot and in a bipedal position facing a wall and will be supported with their dominant hand on the wall and elbow in semi-flexion to maintain balance. The participant will perform plantar flexion with maximum range for all repetitions. The first flexion is executed using the full range of motion, up to the point of support on the metatarsophalangeal joints. At full flexion, the examiner marks the maximum height reached by the participant's head with an adjustable height instrument. This will ensure that the individual performs plantar flexion using full range of motion in every



repetition. A verbal command will be given to start the test and the participant will perform the maximum number of plantar flexions possible, as fast as possible until the point of voluntary fatigue. Measurements include the number of plantar flexions performed during the bilateral calf-raise, total time in seconds, and repetition rate (repetitions per second). These outcomes can then be normalized for differences in participant physical characteristics such as BMI, height, weight and age.