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Randomized Clinical Trial of The Effect of Oral Supplementation with N-acetyl Cysteine and Glycine on Biomarkers of Oxidative Stress and Inflammation in People Living with HIV (PLWH) from the MASH cohort

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

RANDOMIZED CLINICAL TRIAL OF THE EFFECT OF ORAL SUPPLEMENTATION WITH N-ACETYL CYSTEINE AND GLYCINE ON BIOMARKERS OF OXIDATIVE STRESS AND INFLAMMATION IN PEOPLE LIVING WITH HIV (PLWH) FROM THE MASH COHORT

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

DIETETICS AND NUTRITION

by

Alhanoof Al-Ohaly

2020

To: Dean Tomás R. Guilarte
R.Stempel College of Public Health and Social Work

This dissertation, written by Alhanoof Al-Ohaly, and entitled Randomized Clinical Trial of The Effect of Oral Supplementation with N-acetyl Cysteine and Glycine on Biomarkers of Oxidative Stress and Inflammation in People Living with HIV (PLWH) from the MASH cohort, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: March 18, 2020

The dissertation of Alhanoof Al-Ohaly is approved.

Dean Tomás R. Guilarte
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Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2020

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DEDICATION

I dedicate this dissertation to my mother and father, without their moral and emotional support throughout my life and my doctoral degree I wouldn't have made it this far. I shall forever be indebted.

A special feeling of gratitude goes to my eldest brother, Fawaz, without him I would have never been able to pursue my degree. To the only person, my sister Basmah, special thanks for living with me through my toughest times and sharing this beautiful experience. Many thanks to my siblings, Abdulrahaman and Talah, the two pillars of my strength and emotional support living miles away. I also dedicate this dissertation to my grandfather, for his persistent determination to being whatever I want to be.

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ABSTRACT OF THE DISSERTATION

RANDOMIZED CLINICAL TRIAL OF THE EFFECT OF ORAL SUPPLEMENTATION WITH N-ACETYL CYSTEINE AND GLYCINE ON BIOMARKERS OF OXIDATIVE STRESS AND INFLAMMATION IN PEOPLE LIVING WITH HIV (PLWH) FROM THE MASH COHORT

by

Alhanoof Al-Ohaly

Florida International University, 2020

Miami, Florida

Professor Adriana Campa, Major Professor

HIV infection has been associated with glutathione (GSH) depletion, oxidatively damaged DNA, and inflammation. People living with HIV (PLWH) have subnormal levels of GSH and elevated levels of inflammation biomarkers such as C-Reactive Protein (CRP). Failure of the antioxidant enzymatic system increases oxidatively damaged DNA. The objective of this double-blinded randomized clinical trial was to supplement PLWH with a combination of N-acetylcysteine, a powerful antioxidant, and glycine, a precursor of GSH or placebo for three months to decrease oxidative stress and inflammation.

The trial recruited 30 PLWH from the Miami Adult Studies on HIV (MASH) cohort at the FIU Research clinic in the Borinquen Health Center, Miami-Dade, Florida. Participants were on stable ART, have undetectable HIV viral load, CD4 count ≥500 cells/μL, <65 years old, BMI ≤30 kg/m 2, and free of co-morbid diseases. Cocaine was used by 50% of the participants and cocaine users were equally distributed between the

intervention and placebo groups. We collected anthropometric measurements, pill count, CRP, demographics, and blood samples. The alkaline and enzyme modified comet assay was performed in whole blood to assess levels of oxidatively damaged DNA (SB/ALS, SB/ALS + oxidized purines, and oxidized purines only), and GSH was assessed using the Arbor Assay Glutathione Colorimetric. Supplementation significantly reduced levels of AS/ALS + oxidized purines ($p=0.005$) and SB/ALS ($p=0.05$). There was a direct correlation between BMI and SB/ALS ($r= 0.585$, $p=0.009$). Pill adherence was significantly associated with supplementation ($\beta= 0.591$, $p=0.001$), 75% in the intervention group were adherent. Oxidized glutathione (GSSG) decreased after supplementation, yet did not reach significance. Post-supplementation CRP significantly decreased among male gender ($p=0.011$), and was associated with decreased % body fat ($p=0.022$), BMI ($p=0.004$), and GSSG ($p=0.019$). Cocaine use was associated with lower CD4 cell counts ($p=0.045$) at baseline. Supplementing was effective in reducing levels of oxidatively damaged DNA and biomarkers of inflammation, and it also decreased body fat % and GSSG among PLWH from the MASH cohort.

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CHAPTER I: INTRODUCTION

Statement of Problem

Worldwide, HIV remains one of the world's major public health concern, with nearly 37.9 million people living with HIV (PLWH) at the end of 2018, with approximately 1.7 million new infections in the same year.[1,2] In the United States, 37,832 people were diagnosed with HIV in 2018, which suggested decline in new infections; however, there are approximately 1.1 million PLWH.[3] In Miami-Dade, Florida, where this study was conducted, there are more than 27,908 PLWH.[4] Miami-Dade county has one of the highest HIV incidence and prevalence rates in the United States.[3] The number of people living with HIV/AIDS in Miami-Dade County has increased by 23% from 2004 to 2014.[5] Thus, the HIV pandemic is a pressing problem in our community.

The human immunodeficiency virus (HIV) is a retrovirus that destroys and impairs the function of the immune cells.[6] Since 1996, in the era of antiretroviral therapy (ART),[7] HIV has become a chronic and manageable disease, if patients strictly adhere[8] to ART more than 80 to 95% of the time and attend their healthcare visits.[9] Despite stable ART, however, inflammation and immune activation have become major challenges for those infected with the disease with adverse consequences for HIV progression, co-morbid cardiovascular disease, and mortality.[10,11,12]

Significance of Study

During infectious diseases, antioxidant and anti-inflammatory activities are vital for controlling inflammation and oxidative stress.[13] HIV presents change in the components of the antioxidant defense system, resulting in Glutathione (GSH) depletion which may lead to DNA damage. [14] Studies have shown that HIV results in increased oxidative stress. [15,16,17] Increased oxidative stress generates free radicals, which then can damage the cell and produce excessive inflammation biomarkers. The literature is limited in considering all these aspects among PLWH. Besides, novel biomarkers are needed to advance research in this area.

Innovation

This is the first study to apply the alkaline-hoGG1-modified comet assay to HIV blood samples.

Aims and Hypotheses:

Primary Aim: To assess the effectiveness of three-month oral supplementation of N-acetyl cysteine and glycine on plasma GSH concentrations, DNA damage, and biomarkers of inflammation in HIV-infected individuals from the MASH cohort, on stable ART, with controlled viral load. We conducted a double blinded randomized clinical trial of 3-month supplementation.

Hypotheses:

Hypothesis 1: Supplementation with N-acetyl cysteine and glycine in adult PLWH on stable ART with controlled viral load will decrease the ratio of plasma reduced GSH levels to free glutathione.

Hypothesis 2: Supplementation with N-acetyl cysteine and glycine in PLWH on stable ART with controlled viral load will lower inflammation as measured with plasma hsCRP.

Hypothesis 3: Supplementation with N-acetyl cysteine and glycine in PLWH on stable ART with controlled viral load will result in lower DNA damage.

Table 1: Statistical Analysis of Hypotheses

Hypothesis	Independent and Dependent Variables	Outcomes	Controlled Variables	Statistical Analyses
1a: Supplementation with N-acetyl cysteine and glycine in adult PLWH on stable ART with controlled viral load will decrease the ratio of plasma reduced GSH levels to free glutathione.	Independent: Supplementation with NAC and glycine Dependent: Levels of reduced glutathione	Monthly proportion of people increasing reduced glutathione in each group	Age, Gender BMI at baseline, Fat mass, CD4 cell count at baseline Alcohol and drug use at baseline and over time	Primary analysis: Intent-to-treat. Linear Regression was performed to assess the relationship between supplementation and GSH.
1b: Supplementation with N-acetyl cysteine and glycine in PLWH on stable ART with controlled viral load will result in lower DNA damage.	Independent: Supplementation with NAC and glycine Dependent: Oxidatively DNA damage	Monthly proportion of people with reduced markers of inflammation in each group	Age, Gender BMI at baseline, Fat mass, CD4 cell count at baseline Alcohol and drug use at baseline and over time	Primary Analysis: Intent-to-treat. Logistic Regression was performed to assess the relationship between supplementation and DNA damage -
1c:Supplementation with N-acetyl cysteine and glycine in PLWH on stable ART with controlled viral load will lower inflammation as measured with plasma hsCRP	Independent: Supplementation with NAC and glycine Dependent: hsCRP	Monthly proportion of people with adverse events and types of adverse events in each group.	Age, Gender BMI at baseline, Fat mass, CD4 cell count at baseline Alcohol and drug use at baseline and over time	Primary Analysis: Intent-to-treat. Linear Regression was performed to assess the relationship between supplementation and inflammation measured by hsCRP

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CHAPTER II: LITERATURE REVIEW

HIV and Oxidative Stress

HIV contributes to increased oxidative stress in several manners.[1] Oxidative damage occurs when the production of Reactive Oxygen Species (ROS) exceeds the capacity of antioxidant defenses to neutralize them.[2] Oxidative stress damages lipids, proteins and DNA,[3] and measurements biomarkers of this damage can be used to determine the extent of targeted and systemic oxidative stress.[4] To protect tissues and organs against oxidative stress, the body possesses external and internal antioxidant defenses, including glutathione (GSH) and its associated enzymes.[5]

HIV in Miami-Dade

HIV infection leads to increased oxidative stress, which may in turn lead to faster development of HIV-related chronic diseases.[1,6] Hence, antioxidants might have a significant adjuvant role in the treatment of HIV/AIDS.[6Error! Bookmark not defined.] The Miami Adult Studies on HIV (MASH) cohort is recruiting 1,500 participants, half of whom are people living with HIV (PLWH). Miami-Dade County currently has approximately 27,908 PLWH[7] and a concentrated and growing HIV epidemic, which makes the development of interventions to improve their quality of life, prevent and treat co-morbidities and delay mortality are goals for this population.

Glutathione and its Amino Acid Precursors

Glutathione (GSH) is a tri-peptide (L-γ-glutamyl-L-cysteinylglycine) with numerous biological roles such as safeguarding against reactive nitrogen species (RNS) and ROS. [8] Glutathione is the greatest copious low molecular weight thiol compound that is created in the cells,[9] which is crucial in safeguarding cells from oxidative destruction, as well as xenobiotic electrophiles toxicity, and sustaining redox homeostasis.

Glycine (Gly), cysteine (Cys), and glutamate (Glu) are the amino acid precursors needed to synthesize GSH.[10] They are typically categorized as nonessential amino acids; nevertheless, in some cases, the ability to synthesize GSH can be inadequate.[11,12] Amino acids are either essential, non-essential, or conditionally essential.[12] The essential amino acids are driven from the diet and the non-essential are synthesized in the body. However, some of the non-essential amino acid become conditionally essential, to be consumed through diet in the presence of some illnesses. Under metabolic pressure, only glutamate and serine may remain non-essential. The conditionally essential amino acids include cysteine, arginine, glycine, tyrosine, glutamine, and proline.

Amino acids are important to maintain good health through their actions in several systems. Some of the important roles for the non-essential amino acids are DNA synthesis, gene expression, immunity, and contribution to the antioxidant defense system.[13]

Glutathione and its precursor, GSH are produced and have antioxidant functions in several body systems, which includes the gastrointestinal, immune and cardiovascular

systems. Therefore, when we have impaired GSH synthesis, its precursors become conditionally essential to maintain the health of the individuals.

Currently, supplementation with GSH precursors has been found to decrease infection rates.[11] These precursors can assist in protecting against oxidatively generated damage emanating from radiotherapy.

The function of the immune system is optimal when lymphoid cells contain glutathione that is well-adjusted at the intermediate level.[14] Even minute changes in the intracellular levels of glutathione have significant impact on the functions of lymphocytes. Some functions, like synthetic response of DNA, are very sensitive to intermediates of reactive oxygen and are thus favored by high degrees of antioxidant glutathione. Placebo-controlled, randomized experiments demonstrate that treating patients infected with HIV with N-acetylcysteine resulted in a considerable rise in all immunological functions including natural killer cell activity restoration.

Reactive oxygen species, Oxidative Stress, and Antioxidants:

Reactive oxygen species (ROS) includes those molecules that contains free radicals and non-radicals based on their oxidizing capacity and reactivity.[15] ROS are produced via various chemical reactions with biomolecules resulting in oxidative stress. Antioxidants are used in protecting biomolecules from the damaging impact that may be caused by ROS. Previously, research on antioxidants largely sought to understand ROS/free radical reactions with antioxidants employing kinetic methods and biochemical. However, current studies also consider the capacity of antioxidants in modulating cellular

signaling proteins, including transcription factors, secondary messengers and receptors among others. Currently, it is evident that antioxidants are critical for human health conditioning in their bioavailability and dosage.

Typical human biochemical reactions, higher degrees of dietary xenobiotics and increased exposure to the environmental pollutants lead to the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS). RNS along with ROS generate oxidative stress in various pathophysiological environments.[16]

Oxidative stress is also linked to aging; however, causal mechanisms are not fully understood. Sekhar et al[17] explored if glutathione deficiency occurs as a result of decreased synthesis and facilitates oxidative stress in aging. The researchers also explored whether stimulation of glutathione synthesis using its precursors glycine and cysteine can decrease oxidative stress. The study found that in ageing humans, glutathione deficiency develops due to a significant decrease in production. Supplementing the diet with the glutathione precursors glycine and cysteine results in restoration of glutathione concentration and synthesis, while lowering oxidative stress levels and oxidatively generated damage.[17]

The mechanisms that form the basis of clinical and therapeutic application of N-Acetyl Cysteine (NAC) are complicated and remain unclear. Samuni et al.[18] explored the chemistry of NAC and the manner in which it interacts and functions at the cellular, tissue and organ level with the aim of bridging the gap between its chemistry and recognized biological activities.[18] The study conclude that NAC has an antioxidant

activity because of its rapid reactions with CO_3, NO_2, OH and thiyl radicals. NAC is seen to be unique for its effect decrease of disulfide bonds in proteins; therefore, changing the structure and disturbing ligand bonding.

Chronic inflammation is associated with many human diseases. This response does not demonstrate any advantages for human health that are condition specific. A diet characterized by antioxidant products is an approach and an emergent trend for dealing with chronic diseases' inflammatory responses, as well as their associated risk factors.[19] Lu and Yen[19] reviewed the possible molecular signaling of antioxidants, along with the anti-inflammatory impacts of specific dietary phytochemicals from common food. The review sought to support health implications and define the understanding of the researchers on natural bioactive substances with anti-inflammatory and antioxidant characteristics.

La Rowe et al.,[20]through a "double blind placebo-controlled crossover Phase 1 trial" sought to measure the tolerability and safety of NAC in healthy cocaine users. Since that the resultant side-effects were mild, they established that NAC was harmless and well-tolerated among healthy individuals using cocaine.[20] Ultimately, the authors suggest that NAC aids to decrease symptoms linked to cocaine withdrawal.

Oxidative stress, DNA damage and the Application of the Comet Assay to Measure DNA Damage:

Oxidative stress is the imbalance between antioxidants and free radicals in the body.[21] Damaged DNA is a chief consequence of oxidative stress. Deoxyribonucleic acid,

a self-replicating material found in almost all living creatures is the core component of chromosomes. When damaged, the DNA causes structural modifications, such as alterations or breakage of its strand, DNA damage can happen naturally or through environmental factors.[22] The damage can alter the coding properties as well as interfere with cell processes. As Collins et al.[23] highlights, disruption in the normal redox state of cells, on the other hand, can cause harmful effects by forming free radical peroxides that harm the constituents of the cell, including, DNA, proteins, and lipids.[23] Oxidative stress due to oxidative metabolism not only breaks DNA strands but also damages DNA bases.

Biomarkers are essential in the evaluation of oxidative stress and the antioxidant defense system. According to Hashimoto et al.,[24] oxidative stress markers that represent the damage due to oxidation are grouped into three categories.[24]First are antioxidants, which represent the systems that handle and decrease oxidative stress. Second are quantification of reactive oxygen species (ROS), which may be found at the cellular level. Third are markers of oxidative damage caused in RNA/DNA, lipids and proteins. Furthermore, DNA damage is considered one of the most common biomarker to measure oxidative stress.[25]

Oxidative stress among PLWH is higher than in the general population due to the chronic infection and treatment, which may have more than one pathway leading to DNA damage. [26] The increased levels of DNA bases destroyed in HIV-infected people rises the chance to develop Acquired Immune Deficiency syndrome (AIDS).[27] Commonly used tools to measure oxidative DNA damage are PCR-based assays, comet assays, and mass spectrometry-based approaches. Comet assay is a procedure that measures DNA damage

in an individual cell.[4,24,29] The alkaline comet assay is the method of choice for measuring different kinds of DNA damage in cells. It is a gel electrophoresis-based technique, which is sensitive, versatile, and relatively simple to perform. The procedure is a result of the appearance of damaged and undamaged DNA, resembling a comet. Most researchers employ comet's skill to detect DNA single-strand breaks.

Uses of comet assay range from sentinel bio monitoring to DNA damage measurement in exact genomic sequences.[28] The comet assay – modified-enzyme alkaline comet assay allows the cellular proteins to detach from the cell through lysing, after which the DNA can unwind under basic conditions.

Unrepaired DNA injuries can cause genetic variability, which accelerates cancer growth.[5] Recognizing the possible causes of DNA destruction is crucial for maintaining the well-being of HIV people. The in vivo, alkaline comet assay senses DNA destruction. Besides, the procedure requires small amounts of cells.

Briefly, the comet assay is a single cell-gel electrophoresis, measuring different types of DNA damage in cells.[8] Conducting the comet assay takes between two and three days, excluding scoring of the comet assay slides. The assay includes lysis overnight, electrophoresis and neutralization, staining and then scoring under the microscope. In 2016, it was established that the comet assay can be carried effectively with a shorter duration of two days.[29]

Previously, the comet assay occupied a large bench space and the microscope slides were placed in a horizontal sequence with a potential to damage the slides, but

Karbaschi and Cooke[8] changed the procedure after founding that shape and orientation of comets run straight up in the high throughput rack and were similar with the ones that were run horizontally, with no considerable variation in proportion of tail DNA in the vertical and horizontal samples. The study also discovered that electrophoresis can be effectively carried out, to ensure the size and shape of the comets is not affected, with the slides in a vertical position, instead of a horizontal position.

Glutathione Supplementation in PLWH

Glutathione (GSH) is an abundant intracellular antioxidant and it is synthesized from its precursor amino acids: glycine, cysteine, and glutamic acid[30,31,32,33] Decreased availability of these amino acids leads to GSH deficiency, which is frequently observed in HIV [15,34,35] In agreement with this, Sekhar et al.[33] concluded that one of the causes of GSH deficiency is diminished GSH synthesis, which is caused by decreased availability of its precursor amino acids. In addition, several studies suggest that excessive production of proinflammatory cytokines including TNF- α, which generates free radicals that are quenched by GSH, is one of the mechanisms of GSH depletion.[13,14, 36, 37,38] Several studies have shown that PLWH have subnormal plasma levels of GSH[39,40,41] and elevated levels of inflammatory biomarkers including C-reactive protein[24, 42] and tumor-necrosis factor alpha (TNF-α).[42]

Sekhar et al.[33] examined whether inflammation in HIV was linked to GSH deficiency and tested whether GSH improvement could lower the elevated plasma concentrations of hsCRP and TNF-α by using nutritional oral supplementation of glycine

and cysteine.[33] In this study, ten HIV-infected men received oral supplementation of cysteine (0.81 mmol/kg/d) in the form of n-acetyl cysteine (NAC) and glycine (1.33mmol/kg/d) for 14 days. They measured (1) inflammation biomarkers (hsCRP and TNF-α), (2) reduced and total GSH, (3) total body fat, (4) insulin sensitivity, (5) mitochondrial fuel oxidation, and (6) reactive-oxygen species. Key findings of this study were that oral supplementation with cysteine and glycine increased intracellular GSH levels by 41%. Simultaneously, there was a 55.4% decrease of plasma levels of hsCRP and 19.2% for TNF-α. These findings support the possibility of a relationship in rising concentrations of GSH in patients infected with HIV was linked to a considerable and rapid decline in the concentrations of hsCRP and TNF-α. The authors' finding is significant since its implications are therapeutic and mechanistic, as they suggest that GSH deficiency can cause inflammatory paths activation in HIV infection. Additionally, a nutritious routine utilizing n-acetyl cysteine, as a cysteine donor, as well as glycine to create a rise in concentrations of GSH may decrease inflammation in HIV. Although this study indicates that a nutritional approach using NAC and glycine may be beneficial among HIV-infected persons by improving GSH concentration and lowering inflammation, it had several weaknesses. The research was conducted in a small group of only male participants, the length of the supplementation was short, and the study lacked a placebo group.

In another study, Alfonso et al.[43] examined whether NAC supplementation had a potential effect on reducing levels of oxidative stress and inflammation among participants with a history of asbestos exposure but otherwise healthy. Sixty-six male

subjects between the age of 40 and 70 years were randomized into either the supplemented (n=34) or placebo group (n=32). This was a four-month randomized clinical trial, where the intervention group received 1800 mg/day of NAC and the placebo group received tablets similar in appearance and taste. They measured total combined thiols (cysteine, glutathione, cysteinyl, glycine homocysteine), and markers of inflammation (CRP and TNF-α). The researchers did not find any variations between the active and placebo group in serum levels of cysteine, cysteinyl, glycine, glutathione and homocysteine. Additionally, none of the subjects had TNF-α above the limit of detection, besides, the investigators did not find considerable variations in CRP. The findings of this study showed that NAC supplementation did not increase antioxidant capacity or improve inflammation biomarkers. Ultimately, there was no evidence that NAC replenished combined thiols of healthy subjects who have previously been exposed to asbestos. However, the investigators believe that these negative findings may have resulted from low supplementation doses, the clinical conditions to reach physiological replenishment of thiols are not well established in alveolar lavage compared to plasma,[44] and/or biased selection of the subject.[43]

Borges-Santos et al.,[30] on the other hand, were able to show that dietary supplementation with either N-acetyl cysteine (NAC) or glutamine (Gln) have positive effects on levels of plasma glutathione, and its three component amino-acids in PLWH. The study recruited 32 participants in total, 12 PLWH on stable ART, and 20 healthy participants. The 32 participants were randomized to receive either NAC 1g/d or Gln 20 g/d, in addition to their usual diet for seven days. Participants were followed for another 7

days without supplementation as a washout period. The levels of glutathione and its three component amino-acids were significantly lower in the HIV+ group at baseline and under the reference levels of healthy volunteers after the 7-day supplementation. The authors found that patients with HIV demonstrated a higher oxidized GSH and decreased concentration of GSH and other amino acids except for homocysteine. The patients with HIV responded to the NAC through increasing amounts of amino acids that contain sulfur and GSH, and balanced levels of GSH and taurine within the control group. The researchers found that Gln and NAC supplements created a rise in Gln, methionine, taurine and GSH, and lowered serine in HIV+ patients. Additionally, supplementation of NAC resulted in considerable rises in homocysteine and cysteine, while supplementation of Gln created a rise in glutamine concentrations. Gln and NAC supplements were also found to have similar impacts on levels of plasma in Gln, glutamic acid, taurine and methionine.

Similar observations were also made by Nguyen et al.[32] showing that reduced levels of cysteine and glycine in serum affect levels of glutathione. This investigation tested if GSH deficiency can be improved by supplementing with GSH precursors (NAC and Gln) and aimed at improving GSH level, expecting that there will be improvement in fed and fasted mitochondrial fuel oxidation. They recruited eight PLWH men 50 years and older, they are all on stable ART, without AIDS, and free of non-communicable disease. Subjects received 0.81 mmol/kg/d of NAC and 1.33 mmol/kg/d of Gln for two weeks, in an open-labeled design. The study measured insulin sensitivity, body composition, and muscle strength. The investigators used a combination of stable-isotope

tracers, calorimetry, hyperinsulinemic-euglycemic clamp, and dynamometry to measure GSH synthesis, fasted and insulin-stimulated (fed) mitochondrial fuel oxidation, insulin sensitivity, body composition, anthropometry, forearm-muscle strength, and lipid profiles. From the research, the authors found that reduced synthesis leads to deficiency of GSH in the patients, which can be restored through supplementation using cysteine plus glycine. It was also found that GSH enhancement occurred together with significant improvement in fed and fasted mitochondrial fuel oxidation. As such, benefits including dyslipidemia, muscle strength, anthropometry, body composition and insulin sensitivity were observed.

One drawback was that the mean age of the subjects was 56 years and aging is associated with oxidative stress,[45] also it has been seen that supplementation among the older population restores GSH and decreases oxidation.

In conclusion, most studies in the literature have shown that oral supplementation of N-acetylcysteine (NAC), glutamine and glycine in PLWH increased plasma levels of GSH and decreased inflammation. The supplementation period in those who found beneficial effects were mostly short, no longer than fourteen days, and the studies included a small number of participants.[13,15,16,25,26]

In summary, high levels of inflammation in PLWH have been associated with GSH deficiency,[16] and excess mortality.[46] Therefore, testing whether supplementing NAC could increase levels of GSH, and lower the elevated plasma concentrations of CRP

and TNF-α needs to be further investigated for longer period of time as potential targets for intervention.

Oxidatively damaged DNA and Antioxidants supplementation

Failure to repair DNA strand breaks is more common in lymphocytes because they get more rapidly depleted of catalase and/or GSH than other cell types. DNA repair capacity is related to levels of intracellular antioxidant activity, which is potentially diminished in PLWH.[47] Increased levels of oxidative stress increase oxidative damage to DNA, which might influence HIV disease progression.[48]

Nygren et al.[49] measured DNA damage in cultured skin fibroblasts from patients with GSH deficiency. Treatment varied among patients between acidosis, vitamin C and/or vitamin E. They measured DNA single strand breaks utilizing comet assay. They expected that GSH deficiency makes cells more prone to oxidative DNA damage leading to apoptosis and neuronal death; however, that was not observed. Although they establish that GSH might not completely protect damaged DNA, yet they found that high levels of oxidative DNA damage are more prone to ionizing radiation. Therefore, the intrinsic cell protection against oxidative stress is dependent on the background levels of damaged DNA. This study is of significance because PLWH have lower levels of GSH, which may contribute to heightened DNA damage, thatsuggests a potential benefit of supplementation for this population.[50]

Duthei et al. [51] conducted a double-blinded supplement study, on healthy smokers and nonsmoker volunteers for 40 weeks. They aimed to measure antioxidant capacity in

plasma and oxidative DNA damage. Half of each group received either antioxidant regimen (vitamins C and E, and β-carotene), or placebo. Similar to our study, the comet assay was used to measure DNA damage and the rate of oxidized DNA pyrimidines was the specific oxidative damage index. By week 20, a significant decrease among the entire supplemented population, smokers and nonsmokers, in DNA damage was observed. This reveals that antioxidant supplementation protects the DNA from endogenously ROS production. Also, Jaruga et al.[2] investigated the effect of antioxidant vitamins on HIV+ patients on the DNA bases and observed that supplementation reduced the levels of DNA oxidative damage. In this study, ten healthy volunteers (controls) were compared to 30 PLWH drug users and 15 HIV-negative drug users. Only the HIV+ patients were either supplemented with vitamins A, C and E or placebo. Levels of damaged DNA before and after supplementation were quantified from isolated chromatin from lymphocytes using gas chromatography/isotope-dilution mass spectrometry with selected-ion monitoring (GC/IDMS-SIM) to measure levels of modified DNA bases. They observed considerable rises in the quantity of the altered DNA bases in patients with HIV relative to the control cluster. There was a greater concentration of thiobarbituric acid reactive substances (TBARS), while antioxidant enzymes (superoxide dismutase and catalase) activities decreased in the cluster of patients infected with HIV compared to the control cluster. It was also found that supplementation of vitamins led to considerable decline in the degrees of all altered DNA bases in comparison to subjects who were given the placebo. The researchers also observed a decrease in TBARS, along with a reestablishment of the enzymes activity. Antioxidant supplementation resulted in reduction in levels of modified

DNA compared to patients who received placebo As such, the data suggest that treatment using antioxidant vitamins can be beneficial to individuals infected with HIV

Hashimoto et al. evaluated GSH influence on DNA damage and changes in blood chemical parameters among young and aged rats.[5] They measured GSH and DNA damage in the liver and kidneys of rats. GSH was measured using a BIOXYTECH GSH 400, commercial kit, and comet assay for DNA damage. They found that DNA damage was higher in aged rats' liver and kidney compared to young rats. However, they found a negative relationship between GSH and DNA damage, which is conflicting with other findings in the literature.[52]

The researchers[5] found a negative relationship between DNA damage and GSH content in the kidney and the liver. L-buthionine (S,R)-sulfoximine (BSO; 0, 5, 20 mM) that inhibit production of GSH, was given through water, which resulted in considerable decrease in levels of GSH in the kidney, lung, liver and heart without damaging DNA in the organs. The results indicated a negative relationship between DNA damage and GSH during aging; however, GSH depletion because of BSO did not have an impact on DNA damage or levels of blood chemistry under the conditions of the study.

Echevarria et al.[53] investigated the in-vitro effect of ascorbic acid on viral load reduction and protection against oxidative damage in the DNA of HIV-infected T-cells. Oxidative DNA damage was measured by the comet assay. The investigators suggest that exposure to ascorbic acid seems to provide some protection to HIV infected CD4+ T-cells. Researchers found decreased DNA damage in HIV-1 positive T-cells that had been

exposed to ascorbic acid. When concentrations were increased, DNA damage was decreased, suggesting that DNA of HIV-1 T-cells was impacted when treated with ascorbic acid.

It has been documented that chronic HIV infection increases the production of reactive oxygen species (ROS), which damages DNA progressively.[52] The body's antioxidant defense system, of which GSH is an important component, protects the DNA from ROS. [54] Considering the association between GSH depletion and DNA damage, an antioxidant regimen should be considered in PLWH.

In summary, the evidence points to the relationship between depletion of GSH and DNA damage, a condition found among PLWH, as well as showing a relationship of excessive ROS with depletion antioxidant defense system resulting in DNA damage in the general population. In view of these findings, it is important to establish the long-term effect of antioxidant supplementation in chronically infected and treated PLWH to delay co-morbid conditions associated with excessive oxidative stress, such as liver fibrosis, cardiovascular disease, prediabetes, diabetes, and cancers.

HIV, ART and Inflammation

Antiretroviral therapy (ART) availability extends the life expectancy in HIV-infected persons.[55,56,57] However, individuals successfully treated with ART are at higher risk to develop chronic conditions such as atherosclerosis, either because the PLWH are aging or because of secondary effects of the disease or its treatment.[58] This population exhibits greater development of some non-AIDS comorbid diseases such as chronic

kidney disease, liver disease, diabetes mellitus, coronary heart disease and atherosclerosis which are associated with inflammation.[12,59,60,61]

HIV infection, both treated and untreated is associated with chronic inflammatory diseases; thus, biomarkers of inflammation including CRP and TNF- α are elevated. [62,63] Moreover, these inflammation biomarkers are correlated with mortality among HIV-infected individuals.[64] Therefore, it is helpful to examine inflammatory biomarkers to predict potential clinical outcomes.[38,33] Since ART prolongs life expectancy among PLWH, aging in this population is associated with an increased risk of age-related morbidities.[65] Although chronic HIV infection is associated with specific mechanisms that may contribute to comorbidities associated with inflammation, such as bacterial translocation and chronic immune activation, other senescent mechanisms may act in parallel to aggravate its effects.[39] There is a growing evidence indicating the crucial role of oxidative stress and resulting changes in redox state in numerous diseases including those associated with chronic inflammation, including cancer, AIDS/HIV, and aging.[66] Senescence also contributes to oxidative stress, therefore, novel interventions need to be implemented to control both oxidative stress and inflammation in PLWH.

SUMMARY

Inadequate levels of plasma GSH in PLWH are associated with accelerated disease progression, higher susceptibility to co-infection with TB, and diminished survival.[13,14,21 ,67,68,69] Preliminary studies in the literature have suggested that supplementation with oral cysteine as n-acetyl cysteine (NAC), glycine, and glutamine as

L-glutamine (Gln) improves cell and plasma GSH levels.[15,16,47] Furthermore, inadequate GSH concentration is associated with inflammation, oxidative stress, DNA damage, rate of cellular aging, obesity, and insulin resistance.[15,16,70] Most of the supplementation studies with GSH suggest that achieving adequate levels in plasma or cells decreases oxidative stress in HIV infection.[49,50 ,71,72,73,74,75] Nguyen et al.[15] found that HIV infected individuals older than 50 years had significant GSH deficiency. Oxidative stress can contribute to chronic inflammation and the development of a variety of pathologies found in many chronic diseases.[76] Furthermore, elevated levels of oxidative stress seem to be associated with two important cardiovascular risks: metabolic syndrome and inflammation in PLWH.[77]

In summary, the studies of supplementation with glutathione and its precursors on the effect of plasma and cell glutathione and overall oxidative stress and inflammation are small in sample size and short in duration, and the evidence on PLWH on stable ART is lacking. Also, there is no previous or existing literature about conducting comet assay in PLWH to measure the effect of oxidative stress and antioxidant supplementation on damaged DNA. Therefore, implementing innovative interventions to demonstrate the safety and efficacy of these antioxidants is significant and warranted.

Oxidatively damaged DNA is a common measure of oxidative stress. Besides, one of the commonly used methods to measure DNA oxidative damage is the comet assay.[78] GSH plays a major role in scavenging the free radicals that are produced by high levels of inflammation. Once levels of GSH are depleted, the antioxidant defense system

is affected. An important approach to prevent damage to DNA through antioxidant pathways, is that antioxidant supplementation could decrease levels of damage.[79]

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CHAPTER III: JUSTIFICATION AND METHODOLOGY

Justification for the Characteristics of the Design:

a) Why oxidative stress in PLWH in Miami-Dade County: HIV infection leads to increased oxidative stress which may in turn lead to faster development of HIV-related chronic diseases.[1,2] Hence, antioxidants might have a significant adjuvant role in the treatment of HIV/AIDS.[1] The MASH cohort is recruiting 1,500 participants, half of whom are PLWH. Miami-Dade County currently has more than 27,000 PLWH and a concentrated and growing HIV epidemic, which makes the development of interventions to improve their quality of life, prevent and treat co-morbidities and delay mortality meaningful and important research goals.

b) Why supplementation with NAC and glycine: Glutathione deficiency contributes to oxidative stress, which plays a key role in aging, the pathogenesis of HIV and cardiovascular disease.[3] Only a few studies have supplemented N-acetyl cysteine (NAC), glutamine and glycine orally in PLWH, but the reports from these trials suggested beneficial effects on concentration of GSH, which is an important intrinsic antioxidant in cells and in peripheral blood.[4,5,6] Previous supplementation studies, however, were mostly conducted for a short period of time using a small number of participants. Therefore, a randomized clinical trial of supplementation with these antioxidants, for a longer period, with adequate sample size is warranted.

c) Why a randomized controlled trial of supplementation: Randomized controlled clinical trials of nutritional supplementation are considered to be the most reliable design to develop evidence-based interventions,[7,8,9] as long as they have

adequate sample size, internal/external validity and sustained for an adequate time periods.[9]

d) Why measures of oxidative stress: (GSH, DNA damage) Most of the supplementation studies with GSH, and its components, such as N-acetyl cysteine and glycine,[4,5,6] suggest that achieving adequate levels in serum or cells decrease oxidative stress in HIV infection. [10,11,12,13,14,15,16]As a measure of oxidative stress, we selected the ratio of oxidized glutathione to total glutathione and % DNA tail, which have been associated with oxidative stress in other HIV studies.[17,18,19] DNA damage is a common measure of oxidative stress.[20] Besides, one of the commonly used methods to measure DNA oxidative damage is the comet assay.[21] There are no previous studies that conducted the comet assay with HIV infected blood. Al-Salmani et al.[22] developed an advanced protocol, where they decreased the size that the comet assay occupies on the bench.[22] Moreover, this novel method was examined once with whole blood.

e) Why measures of inflammation: Elevated levels of oxidative stress seem to be associated with two important cardiovascular risks, metabolic syndrome and inflammation in PLWH.[23] Two well accepted measures of inflammation that have been used in other HIV-related studies are proposed for this study: serum hrCRP,[24,25,26,27,28] and TNF-α, a pro-inflammatory cytokine.[24,25]

f) Why the selected inclusion/exclusion criteria: will be selected from the MASH cohort because this is a well-characterized cohort with a documented history or medications (types and length of ART), measures of disease progression (HIV viral load and CD4 cell count), and the participants' compliance with study visits is known. Therefore, we can determine during the screening visit their compliance with their

MASH study visits and their measures of adherence to medication such as viral load ≤ 400 copies/mL, and, using this information, exclude those who are non-adherent or non-compliant.

The HIV, HCV and HBV status of MASH participants are also known, as well as their age and BMI. BMI ≥ 30 kg/m^2,[29] age ≥ 65 years of age,[30,31] and participants with Hepatitis B or C[32,33] will be excluded, in order to control for well-known factors associated with increased oxidative stress in this population.[34] To avoid confounding effect of other nutritional supplement, we will exclude participants who are on nutritional supplements. We are also excluding pregnant women who are required to take supplements during pregnancy.[7]

Population, Recruitment, Inclusion/Exclusion Criteria

1. Population:

Subjects for this study were recruited from the Miami Adult Studies on HIV (MASH) cohort at the Florida International University (FIU) Research clinic at the Borinquen Health Center, Miami-Dade, Florida. The study was reviewed and approved by the FIU Institutional Review Board.

2. Intervention:

This study was a double-blinded randomized controlled trial (RCT) with a 3-month intervention period. Participants were randomized to receive either NAC (100 mg/day) and glycine (100 mg/day) by *NOW FOODS* or Placebo, which was cellulose by *Medisca*. Participants visited the clinic at baseline and once a month for three months. At baseline and each visit, blood was collected to measure markers of oxidation. Throughout

the study, participants were asked not to take any other vitamin supplementation. Monthly phone calls were made and pill count was administered to assess their adherence to the intervention regimen, and compliance with clinic visits. The study took place between March 2018 – March 2019.

3. Eligibility and Inclusion/Exclusion Criteria

Inclusion criteria:

1) Age ≤ 65 years old

2) Participating in MASH cohort

3) Able to provide written consent for this study

4) BMI ≤ 30 kg/m^2

5) HIV positive by documentation

6) On stable ART for more than 6 months

7) Free of co-morbid diseases (uncontrolled diabetes, symptomatic cardiovascular disease, free of HCV, HBV as confirmed by test)

8) Undetectable viral load.

9) English speakers only

10) Not on nutritional supplements for the past three months.

Exclusion criteria:

1) Not meeting the inclusion criteria

2) Pregnancy or intention to become pregnant

3) Using nutritional supplements

4) Participating in other blinded clinical trial.

5) Spanish speakers

6) Not willing to participate

4. Recruitment:

Subjects for this study were screened and recruited from the MASH cohort at FIU Research clinic in the Borinquen Health Center, Miami-Dade, Florida. This recruitment strategy has the advantage that the cohort is a well characterized and consists of a large number of PLWH (more than 800 participants), the participants' locator information is available to recruiters for follow-up, and their clinical records are available with the participant's permission.

The study statistician provided a list with the participants meeting the inclusion criteria. For further accuracy the list was assessed extensively for adherence to medications and Cohort visits, spoken language, supplement intake at each visit, and all inclusion criteria before contacting the participants. To ensure that the participants are going to be adherent and compliant with the clinical trial visits and supplements, adherence to the MASH cohort visits and HIV medications were taken into consideration. Moreover, each participant was contacted via phone call or contacted when they come for a Cohort visit, to ensure: they spoke English, they are not on any supplements, and willing to participate. The study was explained thoroughly, on the phone or in person in the clinic. After this further assessment, participants were recruited to the study and their information was sent to the pharmacy to have their pills prepared.

Before contacting the participants and after assessing each participant on the list, their visit dates were checked to know when they will be attending the closest clinic visit to the cohort, and within couple of weeks before the visit, the clinic staff was informed that this participant will be screened after his/her appointment, to allow 10-15 minutes to

explain this study and obtain consent if subject was eligible and willing to participate. If the participant's closest visit was not recent they were contacted via phone for a pre-screening and initial recruitment.

Phone call recruitment: As some of the eligible participants have already visited the clinic for their annual or semiannual visit, they were contacted via phone to confirm their preferred contact information (phone number), then made sure they are not on any supplements for the past three months. After asking all the question and checking if participants met all inclusion criteria, the clinical trial was explained thoroughly and stopped at each section to make sure if participant has any questions. Before confirming that participants were eligible, they were asked once again if they understood the study and if they have any further questions. Finally, an appointment was made for a baseline visit and pill dispensation. Usually, they were given an appointment a week after the phone call to allow the pharmacy to randomize participants and prepare the supplement. The FIU pharmacy was provided with the information needed the same day the phone call was completed.

Clinic recruitment: while assessing the participants' eligibility, some had their cohort visits within the week or in couple of weeks. Hence, they were screened in person and recruited at the same day they come for a cohort visit. They were told about the clinical trial after they completed their cohort visit. Once they agreed to participate, they signed consent document then given an appointment for one week later.

Intervention/Clinical Trial:

A. Randomization:

Following the screening eligible subjects were identified; participants were randomized into either the intervention arm or control/placebo arm. A computer program to perform stratified randomization with the use of a blocked randomization technique was used under the supervision of the study statistician.

This study is a double-blinded clinical trial; therefore, the study statistician pre-randomized 70 subject to one of two groups (1 or 2). Then the pharmacist at the FIU pharmacy defined each group and as they were provided with subjects willing to participate they were placed in the group following a chronological order.

FIU Pharmacy:

The FIU Pharmacy packaged the study pills in a double-blind fashion to reduce the potential for observer and participant bias, and to implement treatment masking.

Study Setting:

The activities for this study were conducted at the FIU Research clinic in the Borinquen Health Center, Miami-Dade, Florida, and in the FIU laboratories where most of the laboratory tests were performed. The FIU Pharmacy randomized and bottled the supplements. Participants were enrolled in this study for three months, with a monthly visit. Hence, they visited the clinic for baseline (BL), visit two (on month into the study), visit three (two months after BL), and visit four (the last visit, three months after the BL)

B. Consent:

Following the pre-consent, phone consent, those participants signed the consent in the clinic when they came for this study baseline visit.

After identifying eligible participants from the list provided, phone calls were made to recruit participants and a pre-consent was conducted. In the phone consent the study was explained thoroughly to participants, asking them about their willingness to participate, if they have any questions, and set an appointment a week from the phone call. Once the participant came for the initiated study first visit/baseline visit, the study was explained again before signing the consent. Some eligible participants were interviewed and consented when they came for a cohort visit and set their appointment for the clinical trial baseline visit.

C. Pharmacy:

Only the statistician and the pharmacist had knowledge of the randomization groups in order to prepare the pills. The pharmacist received the information of each subject willing to participate after the consent process and had the blinded supplement ready for pick up to the clinic. For dispensation.

Vials:

The vials were covered to avoid bias. The pharmacy added minimal general information on the vial. This information was limited, which included: participant clinical trial ID, cohort ID, and date of birth. The pill bottles were stored in the pharmacy where all the pill preparation took place until they were delivered to the clinic.

Pills:

Dispensation:

Pills were dispensed at the baseline visit. Each participant received two bottles with 100 tablets in each. Participants were asked to take one pill from each bottle daily,

with a total of two pills daily. Bottles were defined as bottle A and B, to help the participant differentiate between them.

Pill count:

After the baseline visit, the participants brought the bottles for pill count to ensure compliance. Hence, three pill counts were performed for each participant that completed the study.

Table 1: Variables collected at each visit and variables obtained from the MASH cohort:

Variable/visit	**Baseline**	**Visit 2**	**Visit 3**	**Visit 4 (end of study)**
Glutathione (*Blood)*	X *From MASH cohort*			X
CRP	X *From MASH cohort*			X *From MASH cohort*
DNA damage *(Blood)*	X	X	X	X
BMI	X *From MASH cohort*	X	X	X
Bioelectrical impedance analysis (BIA)	X *From MASH cohort*			X
24-h dietary recall	X *From MASH cohort*			X
Pill count		X	X	X

Variables obtained from the MASH Cohort:

All obtained data from the parent study were obtained from the cohort most recent visit to the clinical trial.

Demographics, Anthropometric measurements, glutathione, CRP, 24-hour recall and cocaine use were obtained from the MASH cohort.

Demographics were available on REDCap and obtained upon the consent of the participants. Including, gender, age, and ethnicity.

CD4+ and Viral Load were obtained from the medical reports that participants provide when they came for a cohort visit. As they were assessed to confirm participants eligibility.

Glutathione and CRP were obtained for the baseline of the clinical trial from the recent cohort visit.

Anthropometric measurements were obtained from the recent cohort visit, which was available from REDCap. Height was obtained using a stadiometer and it was obtained only at the cohort baseline visit. Weight was measured on a scale with light clothing and was measured at each visit. Body Mass Index was measured in kg/m^2. Bioelectrical impedance analysis (BIA), were measured without shoes and socks. Electrodes were placed on the participant's right wrist and hand, and right foot and ankle.

Cocaine use was obtained from the self-reported questionnaire and confirmed by the urine toxicology test.

24-hour dietary recalls were obtained from a self-report questionnaire at the recent cohort visit to the clinical trial baseline.

Variables collected during the clinic visit for this clinical trial:

At each visit approximately 1-3 mL of blood was drawn to measure DNA damage. At the last visit GSH was measured for this study from the blood drawn.

Baseline variables were all available and obtained from the parent study except for the DNA damage (comet assay) which was analyzed from the blood drawn at the baseline visit.

Visit two and visit three: for this study blood was drawn to measure DNA damage, weight recorded to measure BMI, and pill dispensation and pill counts were conducted to assess adherence.

Visit four: blood was drawn to measure GSH and DNA damage, bioimpedance was measured for fat % and BMI, pill count was recorded to assess adherence.

D. Blood draw (venipuncture):

At each visit, including the baseline, blood was drawn by trained personnel.

The study nurse drew blood for each participant at baseline, visit two, visit three, and visit four for DNA damage. Blood was also drawn at baseline and visit four to measure GSH.

E. Compensation

Each participant was compensated $10 at the end of each visit.

F. Blood aliquots and transportation:

Labels:

Labels for all specimens obtained during the clinic visits were printed as the participant arrived to the clinic and included the participant's clinical ID, amount of blood, number of tubes and date.

For baseline and visit four we collected two lavender 1 mL tubes for GSH. For all other visits, we collected eight aliquoted tubes of blood for DNA (comet assay) with 25 µL and two tubes of 50 µL. After aliquoting, the tubes were stored at -20 C for a minimum of 20 minutes then transported on dry ice to transport to the FIU oxidative stress group (OSG) freezer and stored at -80 C until analysis.

G. Laboratory analysis conducted for this study:

I. DNA damage: Comet assay

Material:

- COMPAC-50 High Throughput Comet Electrophoresis System, was purchased from Cleaver scientific LTD.
- Human 8-oxoGuanine DNA Glycosylase (hoGG1), was purchased from Fisher Scientific

The comet assay assays were conducted from February 2019 to May 2019. Prior to conducting the comet assay experiments with the HIV blood samples collected during the clinical trial, the protocol was tested with rabbit blood to ensure that the enzyme modified and alkaline comet assay protocol for whole blood was ready to be implemented in two different laboratories. Each experiment took two consecutive days aside from scoring the slides. All reagents and necessary materials were prepared ahead of time for each experiment. At each experiment not only HIV blood sample were analyzed, but also positive control slides, which were Hacat cells were also analyzed in the experiment. The HaCat cells were prepared by Abdulahdi, member of the OSG. We included four types of Hacat cells: 1) HaCat without treatment or UVB exposure (control), 2) HaCat +hoGG1, 3) HaCat exposed to UVB (0.5 J/cm^2), 4) Hacat exposed to UVB (0.5 J/cm^2)+ hoGG1.

II. Glutathione

Material:

- Arbor Assay Glutathione Colorimetric Kit, was purchased from Fisher Scientific

The baseline results were opt from the closest cohort visit. Besides, the blood drawn at the end of the study was analyzed to analyze glutathione after the completion of the supplementation.

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CHAPTER IV: The Effect of Three-Month Supplementation on Oxidatively Damaged DNA in PLWH from the MASH cohort

Introduction

The antioxidant defense system is altered in people living with HIV (PLWH), causing excessive production of reactive oxygen species (ROS).[1] During the progression of the human immunodeficiency virus (HIV) to its more advanced terminal form the acquired immunodeficiency disease syndrome (AIDS), the antioxidant defense system is altered, and the production of reactive oxygen species (ROS) is increased.[1] The production of excessive ROS leads to DNA damage.[2] It has been observed that PLWH present damaged DNA bases compared with HIV-seronegative, which may be a contributor to the progression of the infection to acquired immune deficiency syndrome (AIDS).[3] Some of the antioxidant defense system functions is to protect the DNA from being oxidatively damaged by ROS.[4] Glutathione (GSH), an important tripeptide that contributes to the antioxidant system, is abundantly available and is considered to have an essential antioxidant role. GSH is synthesized from three amino acid precursors, glycine (Gly), cysteine (Cys), and glutamate (Glu),[5] in some cases, such as acute and chronic infections, these precursors become conditionally essential.[6,7] Supplementation with antioxidant vitamins have been shown to decrease levels of oxidatively damaged DNA among PLWH.[2]

Another antioxidant, N-acetylcysteine (NAC) has several important roles, including energy metabolism, antioxidant, promotes detoxifications, and is used for treating some diseases.[8] NAC has an important role in decreasing toxic states by its

capacity to prevent excessive ROS production.. [1-4,9,10,11] *Ex-vivo* NAC supplementation reduces levels of ROS and protects against the formation of oxidatively damaged DNA.

Lack of glycine (Gly) availability may result in abnormal metabolism of some nutrients, failure of the immune response, and other undesirable consequences on health.[12] Thus, Gly is considered to be a conditionally essential amino acid to support growth.[13] Gly is a precursor for glutathione, and supplementation has been observed to be effective in individuals with diabetes, cardiovascular and inflammatory diseases, obesity, cancer, and metabolic disorders.[14] This amino acid also regulates the immune function and is utilized in several pathways that generate DNA.[13] The aim of this study was to assess the effectiveness of a three-month nutritional supplementation with NAC and Gly on levels of oxidative stress measured by the levels of oxidatively damaged DNA among people living with HIV (PLWH).

Methods

Study Design

This was a three-month double-blinded randomized clinical trial conducted from March 2018 to April 2019. Participants were randomly assigned to receive either NAC (100 mg/day) and glycine (100 mg/day) or placebo. The participants were recruited from the Miami Adult Studies on HIV (MASH) cohort at Florida International University (FIU) Research clinic at Borinquen Health Center, Miami, Florida.

In order for participants to be eligible they had to be HIV-positive, free of co-morbid diseases, younger than 65 years old, BMI ≤ 30 kg/m^2, and not supplemented with nutrients for the past three months. The study was approved by the Florida International University Intuitional Review Board.

The clinical trial took place in the MASH cohort FIU Borinquen research clinic,where the clinical and laboratory data were collected. Participants visited the clinic once a month, for a total of four visits. At each visit, blood was collected by peripheral venipuncture for the Comet assay, and a pill count and adherence questionnaire were administered to assess adherence to the study supplement. Several variables were obtained from the MASH cohort, which included: height, demographics (ethnicity, age, and gender), viral load, CD4+ cell count, bioimpedance markers (% fat) and inflammation biomarkers. Baseline glutathione (GSH), which included total glutathione and oxidized glutathione (GSSG) in whole blood were obtained from the MASH cohort, however, blood for this study was specifically collected to assess levels of GSH at the end of the study, which will be discussed further in the next chapter.

Blood collection and storage

In the clinic, the blood samples were collected by peripheral venipuncture at each visit (2 mL) in EDTA tubes from 30 subjects. This was then aliquoted in eight 20 μL EDTA tubes, and the aliquots frozen at – 20 °C for approximately 20 minutes. Then the samples were transported to the Oxidative Stress Group laboratory in FIU on dry ice and stored at – 80 °C, until analysis

hOGG1-modified comet assay for whole blood

The alkaline and enzyme modified comet assay was adapted from a recently described reports.[15,16,17,18] We analyzed 5 μL of blood that were suspended in 200 μL of a pre-warmed low melting point (LMP) agarose. Then 80 μL was dispended to each half of the pre-chilled (pre-coated with 1% normal melting point (NMP) agarose) microscope slides on a chilling plate (Figure 1), (Cleaver Scientific, Rugby, UK). After removing the

coverslips, the slides were incubated overnight at 4 °C in the lysis buffer (100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris−HCl, pH 10) containing 1% triton X. The next day slides were washed once with cold double-distilled water for 10 minutes. Followed by the enzyme reaction buffer (ERB: 40 mM Hepes, 0.1 KCL, 0.5 mM Na_2EDTA + 0.2 mg/ml BSA, pH 8.00 adjusted with KOH) for 20 minutes. The ERB was prepared as a 10X stock and frozen at -20 °C in aliquots. Then half of the slides were treated with Human 8-oxoguanine DNA glycosylase-1 (hOGG1; New England biolabs, USA); 60 µL of hOGG1 in ERB to a finalconcentration of 3.2 U/mL hOGG1.[17] The other half was kept in double-distilled water for 10 minutes. Following 45 min incubation, the coverslips were removed and all slides were placed in the slide rack then incubated in the electrophoresis tank (Cleaver Scientific, Rugby UK) filled with electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH≥13) for 20 min. Then the electrophoresis was performed at (300V, 700mA, 150W) for 20 minutes. Then slide-racks were removed from the electrophoresis tank and placed in the neutralization buffer for 20 minutes (0.4 M Tris-base, at pH 7.5). This was followed by washing in cold double-distilled water (20 minutes), staining in the dark with the tank lid closed with propidium iodide (2.5 µg/mL, for 20 min), and lastly washed the slides in cold double-distilled water (20 min). Slides were left to dry before analyzing the comets, DNA damage as percentage tail DNA, using Comet IV software, version 4.2 (Perceptive, UK).

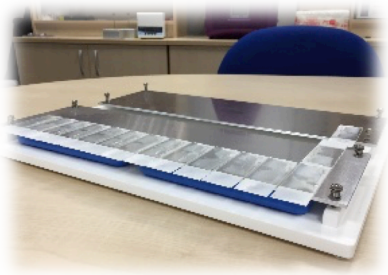


Fig. 1: Pre-coated microscope slides with 1% normal melting point (NMP)agarose) on a chilling plate

Statistical Analysis

Statistical analysis were performed using Prism Version 5 software (GraphPad software) and SPSS 20. The effects of the study supplements were compared using an intent-to-treat analysis initially, and then modified by adherence to the supplements. Descriptive statistics were used to characterize the study population at baseline, and to compare the two study groups. Student's *t*-tests were used to assess mean differences in changes of oxidatively damaged DNA among intervention and supplementation group. Linear regression models were used to determine the relationship between DNA damage and pill adherence, and to assess the effect of supplementation on changes in oxidative stress from baseline to the end of the study. In GraphPad Prism the data were evaluated using paired analysis to compare groups and time points. The level of significance was set at $p<0.05$. Spearman rho and Pearson correlations were used to determine the non-parametric and parametric correlations

Results

A total of 30 participants were enrolled in the clinical trial, with 15 participants in each group. The dropout rate for the three months-study was 37%; 47% (n=7) dropped

out from the intervention group, and 27% (n=4) dropped out from the control/placebo group.

The randomization achieved similar demographic and biological characteristics in both groups at baseline (Table 1). A total number of 30 PLWH were enrolled in this clinical trial, with a mean age of 54.63±6.3 years old, 73.3% (n=22) were Black African Americans. Hispanic and White both represented 13.3% of the population (n=4 each). The population consisted of 66.7% males and 33.3% females. The majority of the population were overweight 70% (n=21). The participants showed 68.4% adherence to the supplement regimen, where intervention group was 75% adherent while the placebo group was 63% adherent to the pills (Table 2).

Supplementation significantly decreased levels of DNA damage (SB/ALS + oxidized purines) in the intervention group p=0.0052 (Fig. 1 C), even levels of SB/ALS p=0.0305 (Fig. 1D). At the end of the study, week 12, oxidized purines only was not significantly different in intervention compared to placebo p=0.1142 (Fig. 1D). We observed similar levels of DNA damage at baseline, as there were no significant differences between intervention and placebo in SB/ALS (p=0.1175, Fig.1 A and B), SB/ALS + oxidized purines (p=0.407, Fig. 1A), and oxidized purines only (p=0.1142, Fig. 1B).

When looking at change in each group from baseline to end of the study we found that supplementation decreased levels of oxidized purines significantly in the intervention group p=0.0055 (Fig. 2 C), and change in SB/ALS was not significant, but approaching significance p=0.0623 (Fig. 2 C and D). There were no significant differences in the

placebo group in SB/ALS + oxidized purines (p=0.9846, Fig. 2 A), SB/ALS (p=0.7535, Fig.1 A and B), and oxidized purines (p=0.7697, Fig. 2 B).

Significant differences in change were observed between intervention and control groups (-4.06±2.8 vs 0.124±3.7, *p*=0.016). The intervention group showed a significant decrease in SB/ALS + oxidized purines from baseline to the end of trial with a p-value<0.05. (Table 3)

A linear regression analysis showed significant association between pill adherence and decreased levels of SB/ALS + oxidized purines *p*= 0.015 (Table 4). Mean levels of SB/ALS + oxidized purines at each one-monthly visit gradually decreased in the intervention group. However, in the control group, we did not observe a considerable change. (Table 5, Fig 3 and 4)

Pearson correlation in the change of measures of markers of oxidative stress and Inflammation

In Table 6, a Pearson correlation was conducted to identify correlations between the changes in oxidative stress and DNA damage, BMI, and % fat, and inflammation. BMI was significantly correlated with SB/ALS (r= 0.585, p=0.009).

Discussion:

In our clinical trial, after three-month supplementation reduced levels of oxidative DNA damage were observed, specifically in SB/ALS and oxidized purines, and in SB/ALS only. We also found that there participants adherent with their supplementation reduced levels of oxidatively damaged DNA, SB/ALS and oxidized purines.

Excessive production of ROS may cause imbalance in the antioxidant capacity of the body antioxidant systems, where DNA and proteins will be exposed to ROS harmful

effects, leading to DNA damage accumulation and amino-acid oxidation.[19,20] Literature shows that HIV infection causes overproduction of ROS and chronic oxidative stress.[2] It has been observed that PLWH suffer from antioxidant deficiency and decreased concentration of antioxidant enzymes, which increases the oxidative stress resulting in oxidative DNA damage. Therefore, supplementing with antioxidants may alleviate the antioxidant depletion.[20]

Our findings indicate that NAC and glycine oral supplementation decreased oxidatively damaged DNA. Our study was similar to that of Jaruga et al,[2] who conducted a clinical trial with supplementing vitamin C, E, and A for the intervention group compared with a placebo group, and fining that antioxidant supplementation decreased levels of oxidatively DNA bases among PLWH. In a study conducted among smokers, [21] it was found that antioxidant supplementation was significant in reducing levels of oxidative DNA damage. Although the population was different, yet, it shows that antioxidant supplementation of vitamin C, E and β-carotene is able to reduce levels of oxidative stress when measured at the DNA level.

Supplementation with NAC was significant in decreasing levels of oxidative DNA damage in human fibroblasts ($p<0.001$) [22], β-thalassemia ($p=0.001$)[20], and mice bone marrow ($p<0.05$).[19] Therefore, interventions in the literature support our findings with the effectiveness of NAC supplementation on oxidatively DNA damage using the comet assay. This supports our findings, therefore, we supplemented with NAC and Gly to assess the effectives of both on levels of DNA damage.

Antiretroviral therapy (ART) prolongs life expectancy among PLWH; however, as they are aging this population is at higher risk for the development of chronic

conditions such as atherosclerosis[23] and other age-related comorbidities.[24] Both human and animal literature shows increased oxidative DNA damage. Older rats have increased oxidative DNA damage in their livers and kidneys when compared with younger rats.[4] In agreement with the literature, the average age of our subjects was 54 years, and we found that oral supplementation of NAC and glycine were significant in reducing oxidatively DNA damage in this aging population. Because PLWH on ART are living nearly normal life spans, the risk of developing chronic diseases similar to those of the general population and rises with aging. Our study shows that a simple and affordable antioxidant regimen may be beneficial in protecting against accelerated antioxidant damage.

We assessed adherence in this study with pill-counts and an adherence questionnaire. Adherence was higher in our intervention group, but the drop-out rate was also higher in this group. Our results in this small sample size, shows that the effect of supplementation was strong over time, and stronger in those who complied with the intervention compared with the placebo group.

Literature regarding glycine as an antioxidant is very scarce. Garcia et al. found that glycine had some effect in preventing DNA damage in a neurodegenerative disorder.[25] While glycine is not considered an effective antioxidant, this study shows it is a powerful free radical scavenger, and its supplementation may strengthen the enzymatic antioxidant system through it role in the formation of glutathione. While NAC is known as an effective antioxidant, it is not possible to determine from this study whether the antioxidant action took place through the actions of glycine or NAC or both compounds as they were supplemented together. However, glycine has been shown to have the ability to protect from oxidative stress by linking protein molecules and form helices. [26]

Strengths and Limitations

The limitations of our study include sample size, large droupout rate, also one of our limitations is that we did not consider assessing glycine and NAC separately, to assess potential synergisms when compared with their combined effect. Strengths of our study, that this may be the first clinical trial to assess the effect of NAC and glycine on oxidative DNA damage in PLWH using the alkaline enzyme modified comet assay.

Conclusion:

Findings of our study show that supplementation with NAC and glycine is beneficial among the HIV population in reducing oxidative stress measured by changes in oxidative DNA damage level over a period of 12 weeks. However, further research is with larger population conducted over longer period of time with a four-arm factorial design (NAC, Glycine, NAC and Glycine, Placebo) to identify the specific effect and assess the magnitude of synergism of the combined group.

Table 1: Baseline characteristics of study participants by groups

Variable	Intervention N=15	Placebo N=15	*p*-value
Age (years)	53.4±6.9	55.8±5.9	0.29
Gender (M) (%, *n*)	84 (12)	53 (8)	0.13
BMI (kg/m^2)	26.92±2.3	25.35±3.7	0.173
Ethnicity(%, *n*)			
African Americans	66.7 (10)	80 (12)	0.322
Hispanic	13.3 (2)	13.3 (2)	
White	20 (3)	6.7 (1)	
CD4+	546±298	577±230	0.759
Viral Load	0.15±0.55	0.066±0.25	0.590
Fat percentage	25.91±5.8	30.12±7.7	0.104
CRP	8.15±16.2	6.02±7.197	0.648
DNA damage (SB/ALS)	4.6375 ±1.95	6.06±2.8	0.118
DNA damage (SB/ALS + oxidized purines)	16.8±2.9	17.89 ±4	0.407
Total Glutathione (GSH)	962.6±361	902.98±237	0.597
Oxidized Glutathione (GSSG)	66.9±37.36	66.8995±38.09	0.999

Data are means ± SD unless otherwise indicated

*Statistically significant, $p < 0.05$

Table 2: Percentage of pill adherence according to the pill count and adherence questionnaire in both groups

	Adherence N= 13 (68.4%)	Non-adherence N=6 (31.6%)
Control	63.6% N=7	36.3% N=4
Intervention	75% N=6	25% N=2

Table 3: Differences in SB/ALS and oxidized purines combined between groups from baseline to the 12-week visit.

Intervention	Placebo	*p*
N= 8	N= 11	
4.0612 ± 2.8	0.124 ± 3.7	0.016

Data are means ± SD. *P* is considered significant at < 0.05

Table 4: Linear Regression Model on the effect of supplementation, age, inflammation and adherence on changes in oxidative stress damage of (SB/ALS and oxidized purines combined) from baseline to the 12-week visit.

Variable	B	95% CI	β	t	p-value
Intervention	-3.533	[-6.21, -0.85]	-.457	-2.824	0.014*
Age	-0.041	[-0.27,0.186]	-0.066	-0.351	0.685
CRP	-0.841	[-0.13, 0.048]	-0.109	-0.590	0.333
Pill adherence	-4.370	[-7.669, -1.769]	-0.532	-2.769	0.004*

Dependent Variable: SB/ALS+ oxidized purines

*Statistically significant

Table 5: Effect of the Intervention and pill Adherence on changes in oxidative DNA damage (SB/ALS and oxidized purines combined) from baseline to 12-week visit.

Variable	B	95% CI	β	t	p-value
Intervention	-3.652	[-6.131, -1.172]	-.472	-3.122	0.007*
Pill adherence	-4.860	[-7.494, -2.226]	-0.591	-3.912	0.001*

Dependent Variable: Difference SB/ALS + oxidized purines from baseline to Visit 4

Table 6: Levels of SB/ALS and oxidized purines combined at each visit by groups

	Control	Intervention	*P*-value
Week 0 *Baseline (Visit 1)*	17.89 ± 4	16.8 ±2.9	0.407
Week 4 *(visit 2)*	18 ± 4.2	15.54 ±2.98	0.130
Week 8 *(Visit 3)*	17.85 ± 3.03	15.69 ± 2.6	0.151
Week 12 *(end of trial/visit 4)*	17.86 ±3.6	13.2 ±2	0.005

Reported as Means ±SD, p-value < 0.05

This table shows levels of DNA damage in SB/ALS and oxidized purines combined, in the intervention and placebo group at all study visits.

Table7: Pearson correlations between study variables

Variables	Fat %	BMI	SB/ALS	oxidized purines	SB/ALS +oxidized purines	CRP
Fat%	1					
BMI	0.036	1				
SB/ALS	-0.141	.585**	1			
Oxidized purines only	0.053	-0.015	0.480*	1		
SB/ALS +oxidized purines	-0.332	0.449	0.765**	0.780**	1	
CRP	-0.331	-0.175	0.209	0.280	0.173	1

*significant at a level of <0.05
*significant at a level of <0.001

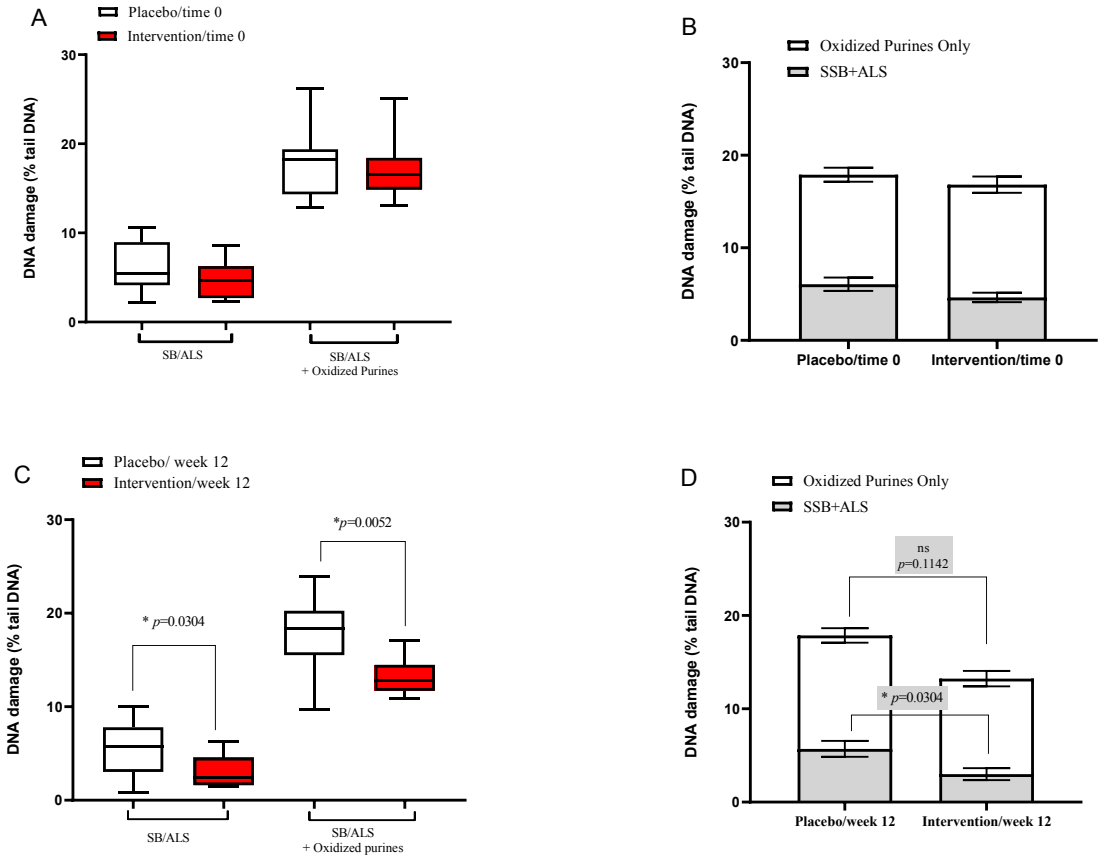


Fig. 1. Effect of supplementation on DNA damage between groups. hOGG1 modified blood comet assay was performed to study blood from HIV-infected individuals pre and post nutritional supplementation. (A) box and whiskers illustrates comet % tail damage with and without hOGG1 at baseline.. (B) means of levels of SB/ALS and oxidized purine at baseline. (C) box and whiskers illustrates comet % tail damage with and without hOGG1 at week 12. (B) means of levels of SB/ ALS and oxidized purine at week 12. .

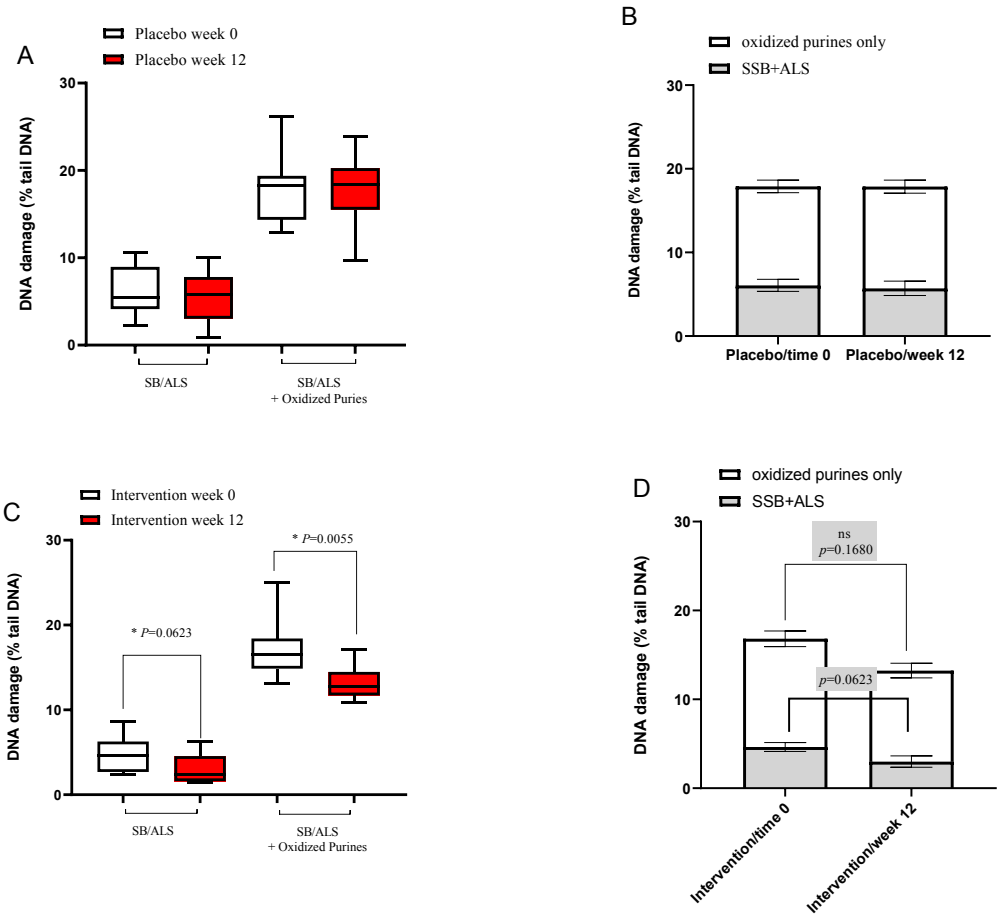


Fig. 2. Effect of supplementation on DNA damage within groups. hOGG1 modified blood comet assay was performed to study blood from HIV-infected individuals and post nutritional supplementation. (A) box and whiskers illustrates comet % tail damage among the placebo group with and without hOGG1 at baseline and end of clinical trial. (B) means of placebo group at baseline and end week 12 levels of SB/ALS and oxidized purine. (C) box and whiskers illustrates comet % tail damage among the intervention group with and without hOGG1 at baseline and end of clinical trial. (B) means of intervention group at baseline and end week 12 levels of SB/ALS and oxidized purine.

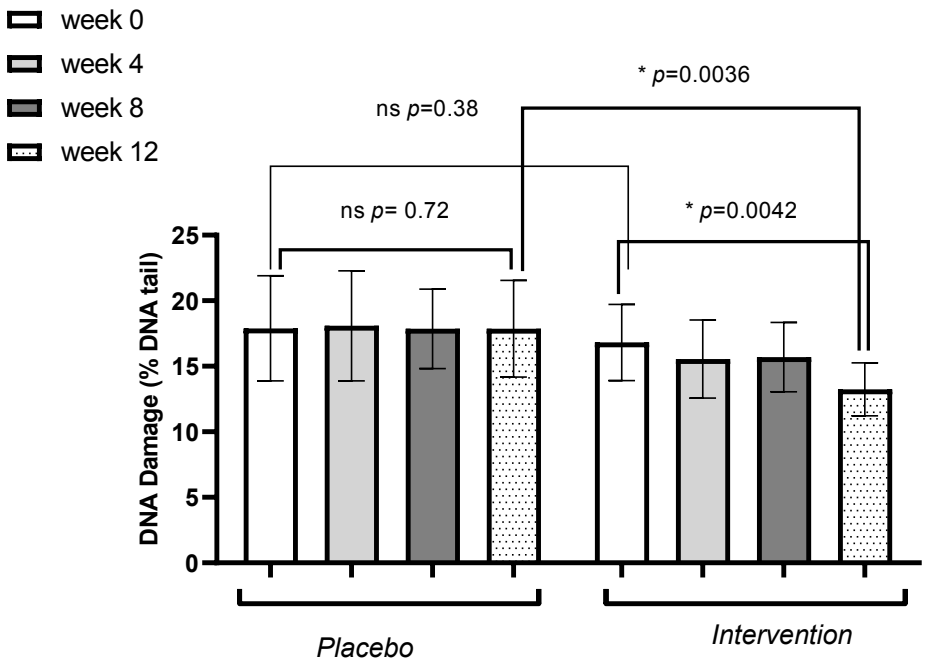


Fig. 3.Total DNA damage between placebo and intervention group at week 0, 4, 8 and 12

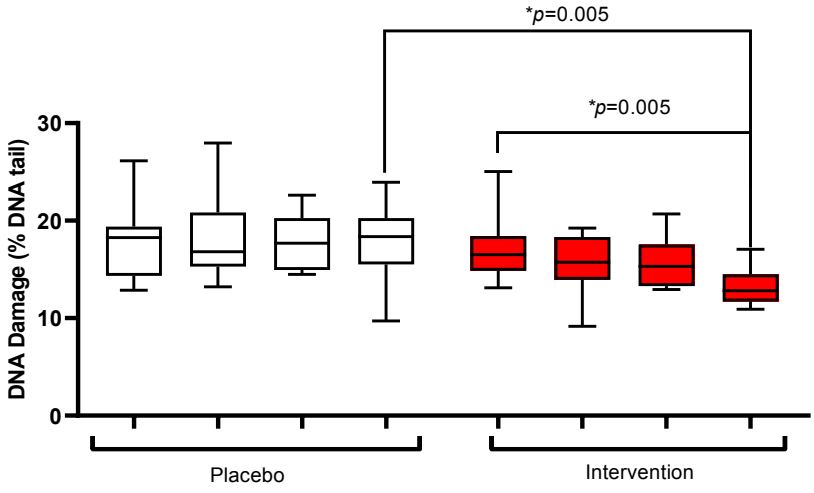
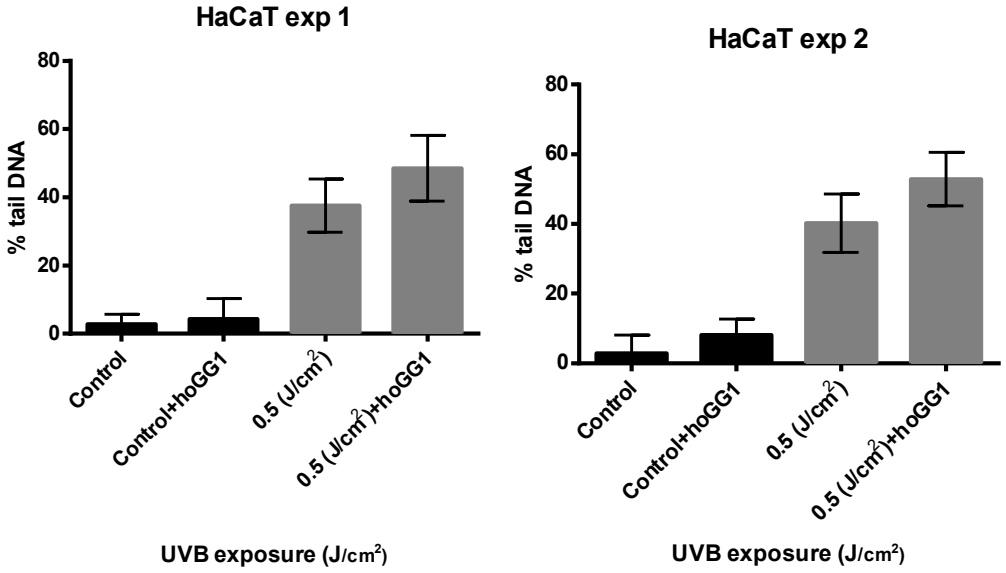


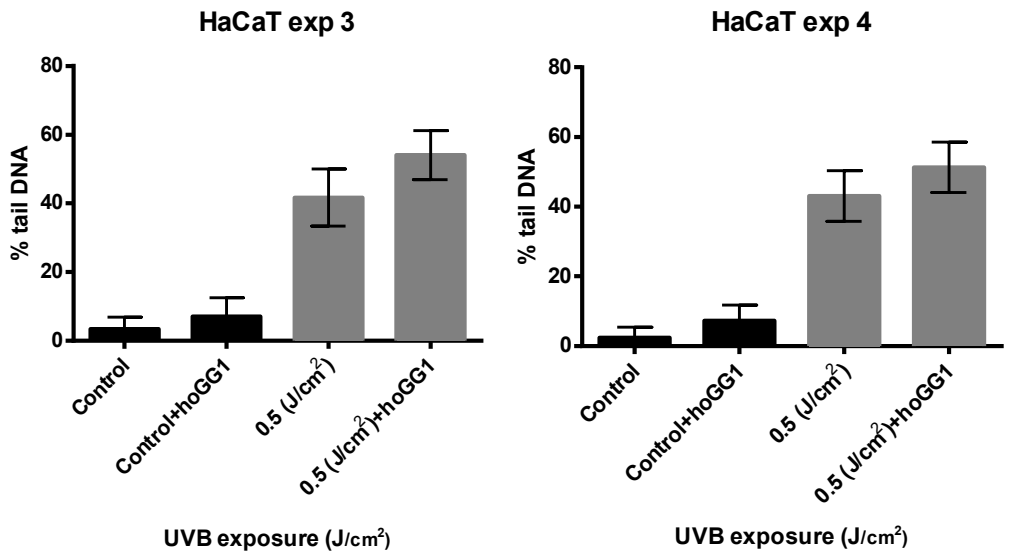
Fig. 3.Total DNA damage, SB/ALs and Oxidized Purines between placebo and intervention group at week 0, 4, 8 and 12

Supplementary Data:

This alkaline comet assay was not tested previously using whole blood from HIV infected individuals. Therefore, to ensure that the comet assay was following the expected conditions we added HaCat cells as positive control to the experiments.

In all the experiments conducted HIV blood samples and HaCat cells were included. We included four types of Hacat cells, which were: 1) HaCat without treatment or UVB exposure (control), 2) HaCat +hoGG1, 3) HaCat exposed to UVB (0.5 J/cm^2), 4) Hacat exposed to UVB (0.5 J/cm^2)+ hoGG1. Our findings showed that the experiments conducted met the expected conditions from techniques, pH levels, and scoring. The figures bellow illustrates bar charts of the % DNA tail for the HaCat cells in four different experiments.





Abbreviations:

- hoGG1: human 8-oxoguanine DNA N-glycosylase 1
- GSH: Glutathione
- GSSG: Oxidized Glutathione
- PLWH: People Living With HIV
- SB/ALS: Strand Breaks/Alkali-labile Sites
- BMI: Body Mass Index

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Chapter V: The effect of NAC and Gly Supplementation on GSH/GSSG in PLWH from the MASH cohort

Introduction

Oxidative stress occurs when there is an imbalance between reactive oxygen free radicals and the antioxidant production. Free radicals contain an uneven number of electrons, which enables them to react freely with other atoms or molecules. These reactions cause damage to the cells and tissues.[1] Antioxidants can donate electrons to free radicals without becoming unstable themselves. This helps in stabilizing the free radicals and making them less reactive.[2] Oxidative stress occurs when the antioxidants are not able to stabilize free radicals.[3] This happens under certain oxidative and inflammatory conditions, such as in people living with HIV(PLWH).

HIV is associated with increased inflammation, which leads to the generation of free radicals. Glutathione (GSH), a tripeptide, is primarily responsible for providing intracellular antioxidant protection against free radicals. [4,5] It is also required for optimal functioning of immune system lymphocytes. GSH exists in two forms; reduced or free GSH performs the antioxidant functions, and GSSG, which is the oxidized form of GSH after combining with free radicals. [6] In PLWH, glutathione levels are lowered, thus leading to oxidative stress. This is because HIV supresses glutathione synthesis by decreasing the availability of its precursor amino acids, namely cysteine and glycine.[7]

Administration of GSH precursor amino acids cysteine and glycine may be a potential solution to address oxidative stress in PLWH as shown previously.[8,9,10] A reduction in inflammatory biomarkers due to increased GSH may bring other health

benefits, since these biomarkers are linked to an increased risk of non-infectious comorbidities such as cardiovascular disease in PLWH.

Sekhar et al[11] found that increasing GSH concentrations in PLWH resulted in a significant decrease in inflammation within a short time. To increase GSH concentrations, nutritional supplements of N-acetyl cysteine and glycine were administered, and the patients tested for levels of inflammation. A reduction of inflammation was indicated by reduced plasma concentration of two inflammation biomarkers: C-reactive protein (CRP) and tissue necrosis factor (TNF-alpha).

According to Gould and Pazdro,[12] oral supplements of GSH are not very effective due to degradation of the tripeptide in the small intestines. However, administration of GSH precursors glutamate, cysteine, and glycine enhances tissue GSH synthesis leading to increased levels for antioxidant protection. Addition of other antioxidant supplements, such as Vitamin C and E, together with GSH precursors further lowers oxidative stress and rate of tissue damage. The other supplements are reported to influence the redox performance of GSH and other antioxidants.[13,14]

Gupta et al.[15] investigated the effects of N-acetyl cysteine on oxidative stress in PLWH receiving antiretroviral therapy (ART). This was a pilot trial to evaluate the potential efficacy and safety of a commercially available supplement using a randomized, double-blind methodology involving PLWH aged 50 years and above. In this population, the oxidative stress caused by HIV is exacerbated by aging. Oxidative stress markers were measured after 8 weeks of administration of the treatment. There was significant increase in levels of reduced GSH and reduction in plasma levels of GSSG. NAC administration also improved endothelial function and was generally

well-tolerated by the patients. Therefore, the aim of this study was to assess the effect of a three-month double blinded nutritional supplementation clinical trial of NAC and glycine or placebo on GSH levels in PLWH from the Miami Adult Studies on HIV (MASH) cohort.

Methods:

The design of this study was a double-blinded randomized placebo-controlled clinical trial (RCT) with a three-month intervention period. Thirty PLWH were recruited from the MASH cohort from March 2018 –March 2019. Recruitment from the MASH cohort has the advantage that the cohort is well-characterized and on-going study and consists of a large number of PLWH (more than 1,100 participants). The aim of this research was to assess the effect of NAC and glycine supplementation compared with placebo in PLWH from the MASH cohort. To be eligible, participants had to be participating in the MASH cohort, HIV status confirmed by documentation in medical chart, on stable ART for more than six-months, have undetectable HIV viral load (VL), CD4 cell count 500 or more, BMI $\leq$ 30 kg/m^2, age $\leq$ 65 years old, free of co-morbid diseases, English speaker, and not using nutritional supplements for the past three months. Florida International University institutional review board approved the study.

Randomization and intervention

Randomization flowchart is shown in **Fig. 1**. The MASH cohort statistician pre-randomized 70 participants to one of two groups and provided the list to the FIU pharmacy prior to subject recruitment. Once potential participants were identified and informed consent was obtained, the participant information was sent to the FIU pharmacy to randomize the participants into either supplementation or placebo groups

following the list provided by the statistician. To avoid observer bias, the vials were blinded by completely covering the contents by the label prior to starting the study. The pharmacist labelled the supplements with a group number and participant identifier following the double-blinded assignment provided by the statistician.

Participants enrolled in the clinical trial were randomly assigned to receive a daily oral supplementation of either one pill from NAC (100 mg/day) and one pill from glycine (100 mg/day) by *NOW FOODS* or two placebo pills, which was cellulose by *Medisca*. Supplements were dispensed during the baseline visit with 100 capsules in every bottle. Each participant received two bottles and was expected to take one pill daily from each bottle. At the end of each visit the participants received an incentive of $10.

Data collection/assessments

Data collection took place in the FIU Borinquen research clinic. Upon consenting participants, demographics (age, gender, and ethnicity), CD4 cell count, viral load, cocaine use, pre-supplementation GSH values and pre-supplementation anthropometrics were obtained from the MASH cohort research charts. Participants visited the clinic once a month; at each visit, blood was drawn for measurements of oxidative stress including parameters of oxidative DNA damage and glutathione status. Concentrations of GSH were assessed at baseline and at end of the clinical trial. A pill count and current weight were obtained at each visit. At baseline and the end of the clinical trial, bioelectrical impedance analysis (BIA) and 24-hour dietary recalls were obtained.

Blood collection and storage

A certified phlebotomist collected blood samples by peripheral venipuncture. Three ml of venous blood was collected and aliquoted in two 1 ml EDTA tubes. The samples were stored at –20 ° for approximately 20 minutes, then blood was transported to the FIU laboratory on dry ice to be stored at – 80 °C until they were analysed in batch.

Laboratory Determination of Total Glutathione and oxidized glutathione

Total glutathione and oxidized glutathione were measured in whole blood by the *Arbor Assay Glutathione Colorimetric*, samples were analysed in triplicates.

Statistical Analysis

All statistical analyses were performed using SPSS (Version 21). Student *t*-test was used to determine differences in mean values. Linear regression was used to determine association of glutathione levels with other factors. Pearson correlation was conducted to correlate measures of oxidative stress. For all analysis significance levels was set at $p< 0.05$ level. Data was normally distributed (Fig 1).

Results

From the MASH cohort a total of 30 participants were randomly assigned to either intervention or placebo group. Nineteen participants completed the three-months clinical with eleven in the placebo group and eight in the intervention group. The majority of the participants were males 66.6% (n=20), with mean age of 54.63± 6.305 (Table 1). Most of the participants were African American 73.3% (n=22). In this population, 70% (n=21) were overweight.

Table 2 shows baseline characteristics by group. In both groups there was more men, 84% in the intervention and 53% in the placebo. African Americans comprised the majority in both groups, 66.7% (n=10) in the intervention and 80% (n=12) in the placebo. There were no significant differences in demographic characteristics between placebo and intervention group at baseline (Table 2).

In addition, there were no significant differences in levels of total GSH and oxidized glutathione at baseline (Table 3). However, at the end of the study, we observed decreased means of GSH in the intervention group. Total GSH decreased in the intervention group by -36.98 μM and increased by 157.2 in the placebo group, p=0.15. Oxidized glutathione decreased in the intervention group by -6.27 μM, and in the placebo group increase by 1.1, p=0.75. Free glutathione decreased in the intervention by -30.7 and increased in the placebo group by 156, p=0.19.

In table 4, we compared levels of GSSG and % GSSG pre- and post-supplementation on GSSG and %GSSG in only the participants who completed the study (n=19), in the intervention and placebo group. We observed no significant changes pre and post supplementation in GSSG and %GSSG in both groups. However, the intervention group had a higher GSSG mean pre-supplementation (81.5389±42.5) compared to post supplementation 75.26736± 41.67μM

In a linear regression model, using change in GSSG as our continuous dependant variable (Table 5), there was a significant association between oxidative DNA damage (SB/ALS) and change in GSSG (β= 0.541, 95% CI= 1.835, 17.859, p=0.020). In the final model, group, cocaine use and pill adherence were included as adjusting variables, as they were the only variables that showed some effect on the

relationship of supplementation and changes on oxidative after three months of supplementation.

Correlations between markers of oxidative stress:

At baseline, levels of GSH were significantly correlated with oxidative DNA damage but not with BMI and body fat percentage. (Table 6) Both GSSG and percent GSH were associated with oxidative DNA damage (r=-0.452, r= -0.422, $p < 0.05$, respectively)

We further found in a two tailed Pearson correlation between GSSG and oxidatively DNA damage (Table 6). ALS/SB were significantly correlated with GSSG (r= -0.410, $p < 0.05$). ALS/SB + oxidized purines were significantly correlated with GSSG (r= -0.452, p=0.012). ALS/SB + oxidized purines were sig correlated with %GSSG (r=-0.4522, p<0.05)

Discussion

This randomized controlled clinical trial (RCT) presented decrease in levels of glutathione (GSH and GSSG). Also, after three months of supplementation decreased levels of GSSG as significantly associated with decreased levels of DNA damage (AB/ALS). Our findings showed evidence that supplementation with NAC and glycine compared to placebo has an effect on reduction of glutathione concentrations among PLWH from the MASH cohort, but these changes did not reach significance. Participants randomized into the intervention in this study were taking a daily dosage of 100 mg of NAC and 100 mg glycine for a total of a twelve-week intervention. Our results compare with those of Alfonos et. al,[16] who supplemented a much higher dose of 1,800 mg of NAC to people exposed to asbestos, using an RCT design, and found

no effect on markers of oxidative stress. Our lack of significant results may be due to a dose of supplementation that was too low to improve levels of GSH, compared with what Alfonso et al. supplemented, however, other studies in PLWH have reported effective doses similar to ours (0.81 mmol/kg/d of NAC and 1.33 mmol/kg/day of glycine in HIV subjects). [11,17] Another factor to consider along with dosage, is the treatment period of supplementation. It was established that NAC supplementation from 12 -24 weeks was beneficial among bronchitis patients,[18] which is similar to our 12- week intervention. However, a chronic infection such as HIV, with a chronic treatment, generates higher oxidative stress than short-term infections, which may explain the differences in results between these two studies. In addition, we have an aging population, and oxidative stress is increased with aging.[19]

The most relevant study to compare our findings with is a study conducted by the previous MASH cohort. [20] It was found that the average GSSG in PLWH was 217.6 μM and in this RCT levels of GSSG was 66.9 μM at baseline. While they reported that %GSSG was 22.52, and in our study, it was 7.43 μM . The levels of GSSG and %GSSG were much lower in our study compared to the findings from the previous MASH cohort study, which indicate that the participants in the current study may have less acute oxidative stress than those found in previous years, probably due to advances in viral control and less toxic medications. Our cohort was selected because adequate viral suppression and functional immune system, both factors that may be contributing to less oxidative stress at baseline.

NAC supplementation has been found to be successful in improving GSH deficiency, especially among individuals with infectious diseases.[21] NAC acts as an antioxidant[22] as it replenishes levels of thiols resulting in improving the antioxidant

capacity. In this regards there is a possibility that our supplementation regimen improved the antioxidant capacity causing a specific effect on other tissues, as we have found in our previous chapter that supplementation was very effective on the level of oxidative DNA damage, including SB/ALS and SB/ALS+ oxidized purines. Therefore, it is possible that supplementation was acting specifically as an antioxidant on other tissues but not specifically in the enzymatic action of glutathione. It would be helpful if we were able to measure and identify other oxidized metabolites.

Although our findings may not reach significance, yet, there was a positive change in GSH levels. Our results, however, show decrease in GSSG in the intervention group when we compare oxidation levels with the placebo group. (Figure 3), there was a decrease by – 6.27 μM in the intervention group, while the placebo group increased 1.1 μM M after three months (Fig. 3). Moreover, % GSSG in the intervention group slightly decreased more than percentage in the control group. (-0.7998±4.5, 0.1283±7.516, respectively) Hence, supplementation with NAC and glycine help reduced levels of GSSG. The lack of significance may also be explained by the small sample size.

In our population regardless of supplementation, there was a significant correlation between GSSG and ALS/SB DNA damage p=0.012, which suggests that by reducing oxidized glutathione, DNA damage may be reduced.

It has been previously established that oral supplementation of N-acetylcysteine (NAC), glutamine and glycine in PLWH increased plasma levels of GSH and decreased inflammation.[11,17,23] Findings from this study are not totally parallel to the literature, as levels of GSH improved but not significantly. Oxidative stress observed in these participants can be attributed to the HIV disease, obesity and

aging, which are important factors that influence oxidative stress. [14,24] Most of our subjects were older than 50 years obese and HIV infected.

There is evidence that supplementation with glycine and NAC was effective among male PLWH [11]as well as older PLWH.[17] In our study, we expected to see similar findings, we looked for gender differences but especially in the intervention group, the majority of the subjects were males, which made findings sexual differences difficult. Our population had an average age of 54 years, and when dividing the population according to the CDC criteria[25,26]for aging, the majority of our participants were in the aging group (83.3%, n=25), however, if we follow the IAS [26] criteria to consider aging, the majority are under the cut-off of aging (73.3%, n=22) are younger than 60 years and only 26.7%(n=8) are aging. Therefore, if we categorize our population according to the CDC our population falls at the beginning of the aging process, however, according to the IAS criteria, our population is not suffering the rigors of aging, including accelerated oxidative stress, yet.

In conclusion, results from this randomized double-blinded trial suggest that supplementation, although demonstrating a protective effect measured by oxidized glutathione, did not have a significant effect on glutathione status, a result that might be affected by the small sample size, the dosage, and/or adequacy of glutathione levels and its precursor amino-acids at baseline in this population. Therefore, the strong relationship between a chronic HIV infection and levels of glutathione found by other studies was not replicated in this study of supplementation, and, although the changes in glutathione levels follow the direction of our hypothesis, they did not reach significant differences between the two study groups.

Strengths and Limitations:

Limitations to this study were sample size with a large dropout rate, as we had only a small number of participants finishing the study, relatively short duration of the clinical trial, and potentially low dosage of the supplements.16 The dosage administered was lower than other studies that had populations with different conditions, and that found that supplementation for a shorter time was beneficial[11,17,18] and the specificity of the population after applying the inclusion/exclusion criteria, which provides for low generalizability.

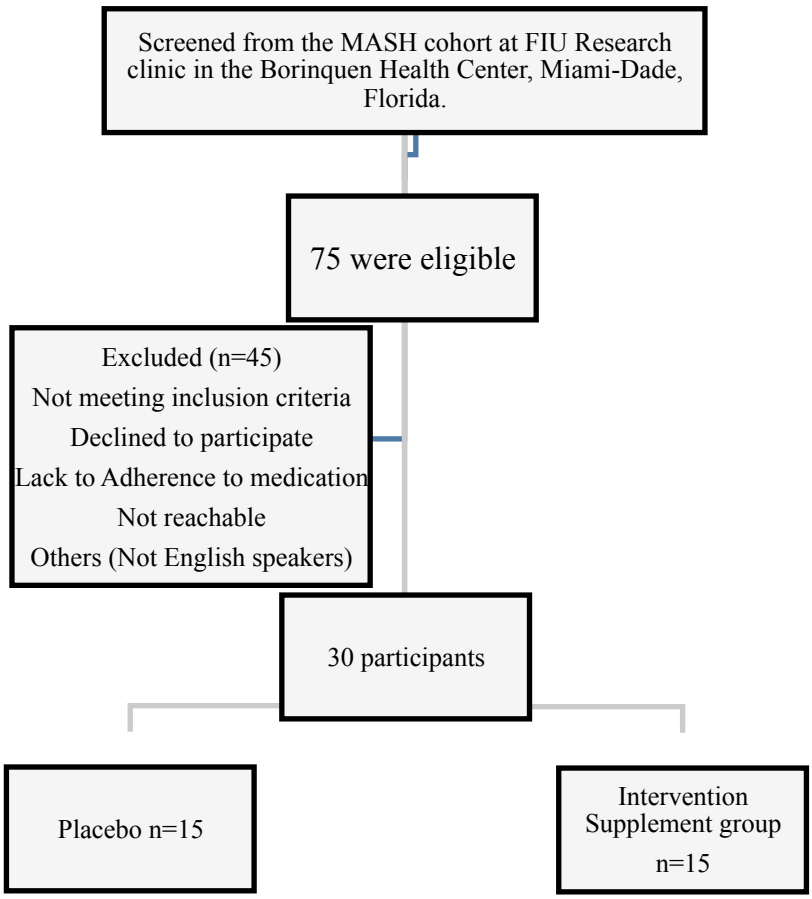

Figure 1. Flowchart of Randomization

Table 1: Baseline Demographics

Variable		
Age		54.63± 6.305
Ethnicity %(n)		
	African American	73.3% (22)
	Hispanic	13.3% (4)
	White	13.3% (4)
Gender %(n)		
	Male	66.7% (20)
	Female	33.3% (10)
BMI		26.13± 3.131
BMI categories %(n)		
	Underweight	3.3% (1)
	Normal weight	20% (6)
	Overweight	70% (21)
	Obese	6.7 (2)

Data are means ± SD unless otherwise indicated

Table 2: Characteristics of study participants by group

Variable	Intervention N=15	Placebo N=15	*p*-value
Age (years)	53.4±6.9	55.8±5.9	0.29
Gender (M) (%, *n*)	80 (12)	53 (8)	
BMI (kg/m^2)	26.92±2.3	25.35±3.7	.173
Ethnicity(%, *n*)			
African Americans Hispanic White	66.7 (10) 13.3 (2) 20 (3)	80 (12) 13.3 (2) 6.7 (1)	
CD4+	546±298	577±230	.759
Viral Load	0.15±0.55	0.066±0.25	.590
Fat percentage	25.91±5.8	30.12±7.7	.104
CRP	8.15±16.2	6.02±7.197	.648
DNA damage (-hOGG1)	4.6375 ±1.95	6.06±2.8	.118
DNA damage (+hOGG1)	16.8±2.9	17.89 ±4	.407
Total Glutathione (GSH)	962.6±361	902.98±237	.597
Oxidized Glutathione (GSSG)	66.9±37.36	66.8995±38.09	.999

Data are means ± SD unless otherwise indicated

Table 3: Change Glutathione concentrations by groups

	Intervention	Control	P-value
Total GSH	-36.9844±322	157.2491±240	0.15
GSSG	-6.27±42.57	1.11 ± 54.5	0.75
% GSH	-0.7998±4.5	0.1283±7.516	0.761
Free GSH	-30.7±350	156±249	0.191

All values are represented as mean ± SD. Data was analyzed using Student t-test. *Statistically significant, $p < 0.05$

Table 4: A comparison of GSSG and % GSSG in control and placebo groups at baseline and end of study

Variable	N	Control	N	Intervention	p-value
Baseline GSSG	15	66.89 ± 38.096	15	66.91 ± 37.36	0.999
BL_% GSSG	15	7.2583 ± 3.53	15	7.60 ± 4.0472	0.805
V4 GSSG	11	70.42 ± 38.38	8	75.26 ± 41.67	0.797
V4 %GSSG	11	7.85 ± 5.37	8	6.97 ± 3.53	0.693

Data are means ± SD unless otherwise indicated

Table 5: Linear Regression model of the effect of supplementation on changes in GSSG from baseline to visit 4.

Variable	B	95% CI	β	t	p-value
Intervention	-0.726	[-43.73, 42.27]	-0.008	-0.036	0.972
Cocaine use ..	-22.595	[-62.560, 17.59]	-.238	-1.147	.271
Pill adherence	26.435	[-18.536, 71.407]	0.259	1.261	0.228
Baseline SB/ALS	9.847	[1.835, 17.859]	0.541	2.636	0.020

*Statistically significant, $P< 0.05$, Dependent variable= change is GSSG

Table 6: Pearson Correlation at Baseline

	Total GSH	GSSG	% GSH	Free GSH	BMI	Fat%
Total GSH	1					
GSSG	.246	1				
% GSH	-.222	.829**	1			
Free GSH	.993**	.126	-.331	1		
BMI	.156	.072	-.075	.151	1	
Fat %	.099	-.050	-.159	.107	-.069	1
SB/ALS + Oxidized Purines	-.343	-.452*	-.422*	-.294	-.038	.250

** Significant at p< 0.01 level (2-tailed), *. Significant at p< 0.05 level (2-tailed), N=30

Table 7: HIV aging Cut-off age according to the Disease Control and Prevention (CDC) and the International AIDS Society (IAS):

Category		Percent (n)
CDC	< 50	16.7 (5)
	>=50	83.3 (25)
IAS	< 60	73.3 (22)
	>= 60	26.7(8)

Data represented as frequencies

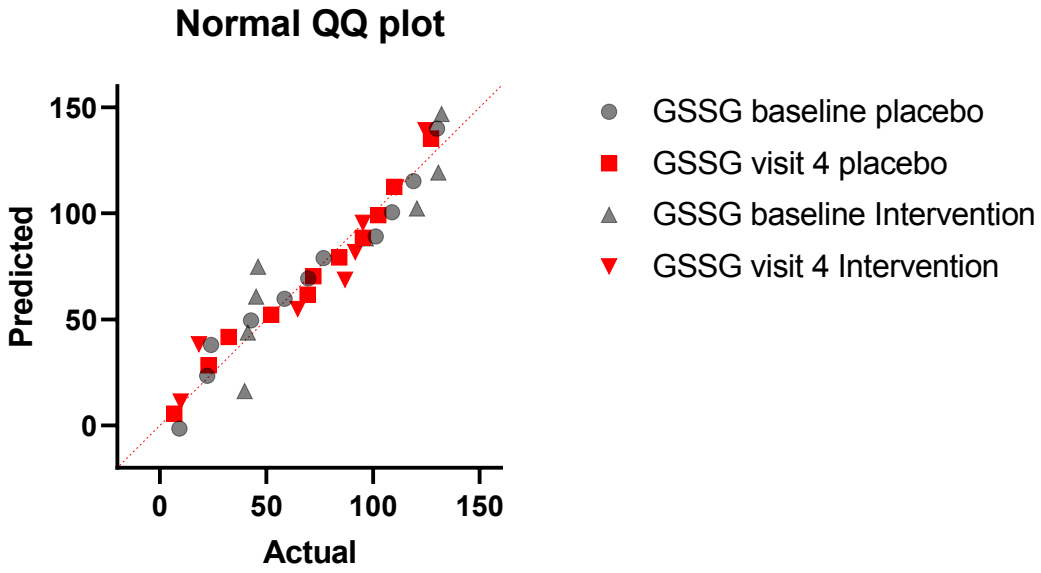

Fig. 1. Oxidized Glutathione (GSSG) Normality test

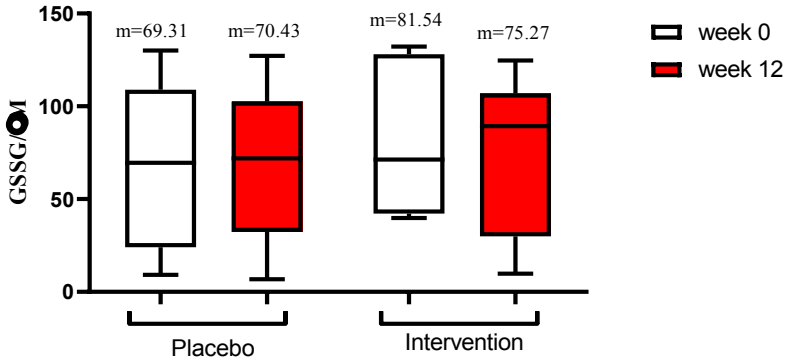

Fig. 2.Differences in oxidized glutathion (GSSG) pre/post supplementation in placebo and intervention group Supplemenetation with NAC and Glycine decreases GSSG by an average of 6.27 mM

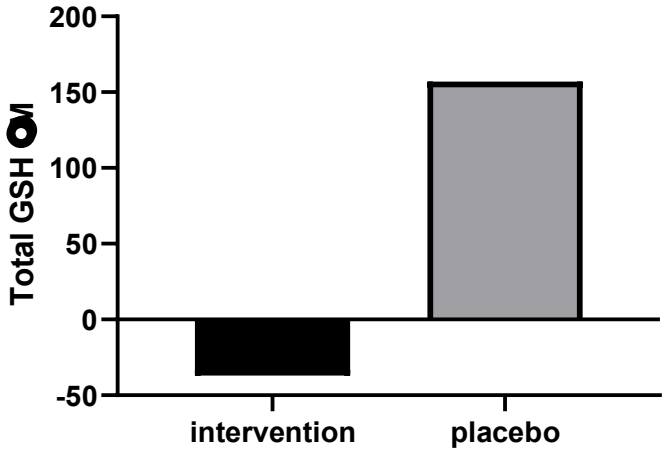

Fig. 3. Change in Total GSH after three months supplementation

Intervention -36.9±322 and placebo 157.2491± 240
Values Represented as means ± SD

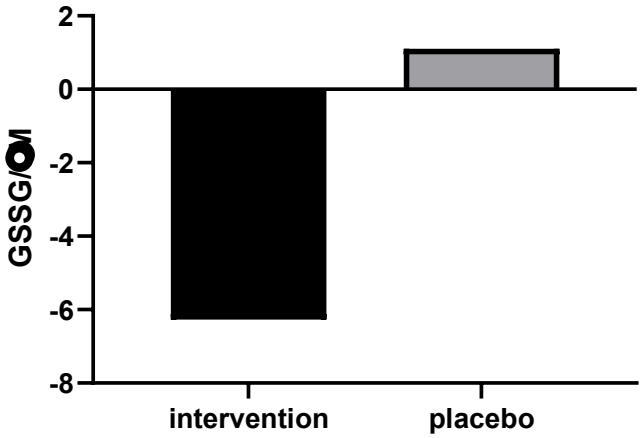

Fig. 4. Change in oxidized glutathione (GSSG) after three months supplementation

Intervention -6.27±42.57 and placebo 1.11 ± 54.5
values are represented as mean ± SD

Fig. 5 Changes in oxidized glutathione (GSSG) after three months of Supplementation

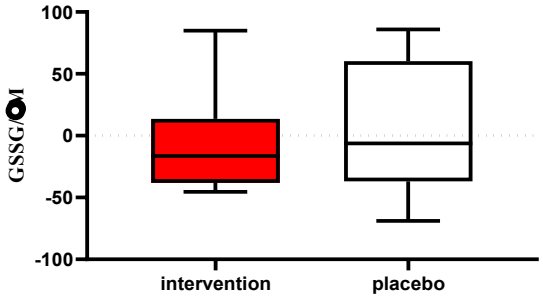

Fig. 1. Change in oxidized glutathione (GSSG) after three months supplementation
Supplementation decreased GSSG by -6.27 in the intervention group while the placebo had an increased 1.1

Abbreviations:

GSH: Glutathione

GSSG: Oxidized Glutathione

PLWH: People Living With HIV

SB/ALS: Strand Breaks/Alkali-labile Sites

BMI: Body Mass Index

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Chapter VI: Cocaine Use, Inflammation, and adherence among PLWH from the MASH cohort

Introduction

Inflammation is associated with many human diseases. A diet characterized by antioxidant products is an emergent approach for dealing with inflammatory responses in chronic diseases, as well as addressing some of the associated risk factors such as obesity.[1] Inflammation is part of the defense mechanisms of the human body; it usually starts with the process of healing as a response to irritants and damaged cells.[2] Zican et al.[3] reported, that despite highly effective antiretroviral therapy (ART), people living with HIV (PLWH) face issues of chronic inflammation or immune activation.

In this regard, high sensitivity C-reactive protein (hs-CRP) has become the most widely utilized inflammation biomarker.[4] Cocaine use is highly prevalent among PLWH in Miami, where our study took place. We have found in our previous studies that HIV infection is an important contributor to persistent oxidative stress, measured by the rate of oxidized glutathione (GSSG) and reduced glutathione (GSH).[5] GSH is a tripeptide, formed by three amino-acids cysteine, glycine, and amino glutamine.[6] Oxidative stress takes place when there is an imbalance between the formation of reactive oxygen species (ROS) and the availability of internal and external antioxidant defenses. PLWH, due to their chronic infection and its treatment, experience oxidative imbalances. In addition, Dehal et al.[7] and Reingold et al.[4] have shown that cocaine use in this population is associated not only with oxidative stress[7] but also with inflammation.[5] Thus, our study of supplementation collected data on factors such as cocaine use, markers of inflammation, and adherence to the supplement to determine

the effects on oxidative stress. In addition, we have used these data to assess the relationship among these factors, with and without the effects of supplementation.

Specifically, to reduce the impact of HIV-infection and its treatment on oxidative stress, in our prior chapters we assessed the effect of N-acetylcysteine (NAC) and glycine as an adjunct therapy, the clinical trial found that this combination was effective to reduce oxidative stress as well as safe and well-tolerated in agreement with previous trial.[8] It has been noted, that oxidative stress is significantly associated with morbidity and mortality among the PLWH but there is limited information regarding the potential multiple mechanisms of action.[9] This chapter focuses on C-reactive protein as a marker of inflammation and the effect of supplementation and cocaine use on inflammation. Cocaine use was equally distributed between the intervention and placebo study groups. Therefore, we explored the effect of NAC and glycine supplementation on inflammation and the role of cocaine use in PLWH from the MASH cohort.[10,11]

Methods

Study Design

In a double-blinded randomized controlled clinical trial (RCT), we recruited thirty PLWH from the Miami Adult Studies on HIV (MASH) cohort at Florida International University (FIU) Research clinic at Borinquen Health Center, Miami, Florida. This was a three-month intervention cohort from March 2018 –March 2019. Subjects were randomly assigned to receive either NAC (100 mg/day) and glycine (100 mg/day) or Placebo. The study was approved by the Florida International University Intuitional Review Board.

Subjects

Participants were eligible to participate if they were HIV-positive, free of co-morbid diseases, on stable ART for more than six-months, have undetectable HIV viral load (VL), CD4 cell count 500 or more, younger than 65 years old, BMI ≤ 30 kg/m^2, English speaker, and not on nutrient supplementation for the past three months.

Data Collection

During the clinical trial participants visited the clinic once a month for three months with a total of four visits. Pre-supplementation data were collected from the MASH cohort research charts, which included bioimpedance body composition measures and biomarkers of inflammation (hs-CRP and TNF-α). At each visit a pill count and adherence questionnaires were collected, weight was obtained, and blood was drawn for outcome biomarkers. Cocaine use was obtained from the MASH cohort reports, as some participants were characterized as cocaine users or non-users according to the participants' self-reported questionnaire, urine toxicology, and cocaine blood biomarkers. We used the MASH cohort characterization for identifying cocaine users and non-users.

High Sensitivity C-reactive protein

Data on inflammation was obtained from the MASH cohort documentation for pre and post supplementation from the closest cohort visit. In the clinic, at each cohort visit, fasting blood was drawn by a certified phlebotomist by peripheral venipuncture. Blood samples were then transported to the HIV laboratory at FIU, where the samples

were collected by LabCorp for analysis and provided the results to the MASH cohort and documented.

Pill count and adherence

At the baseline visit pills were dispensed, with two bottles for each participants for the entire study. Intervention group had two bottles one with NAC pills and one with glycine pills, and the placebo group bottles both had placebo pills. Participants were asked to bring their bottles at each visit for a pill count. Each bottle had 100 pills, and each participant was expected to take one pill from each bottle daily. One bottle was labeled with the letter A and one with the letter B. To assess participants adherence, we calculated [# of pills given - # left] x100, an average for both bottles and 80% was considered the cut-off for adherence. Participant were considered adherent if they scored more or equal to 80% and non-adherent if they had a score less than 80%.

Statistical Analysis:

Statistical analyses were performed using SPSS 20. The effects of the study supplements were compared using an intent-to-treat analysis initially, and then adjusted by rate of adherence to the supplements, cocaine use, and hs-CRP levels. Descriptive statistics were used to characterize the study population at baseline, and to compare by supplementation groups, cocaine use, hs-CRP, and pill adherence. Student's *t*-tests were used to assess mean differences in changes of CRP among intervention and supplementation group, and to assess mean differences of biomarkers of oxidative stress and inflammation among cocaine users and non-users. Linear regression models were used to determine the relationship between CRP, fat %, and

gender, and to assess the effect of supplementation on changes in inflammation from baseline to the end of the study. Spearman rho and Pearson correlations were used to determine the non-parametric and parametric correlations

Results

This clinical trial had a total of 30 participants, with 15 participants in each group. Dropout rate was 37% for the total population, 47% (n=7) dropped out from the intervention group, and 27% (n=4) dropped out from the control/placebo group.

Randomization achieved similar baseline demographic and biological characteristics in both groups (Table1). Most of the participants were males 66.7% in the intervention group and 33.3% in the placebo group, with a mean age of 54.63±6.3. The majority of the participants were overweight 70% (n=21). African Americans represented 73.3 %(n=22), Hispanic and White both represented 13.3% of the population (n=4 each).

At baseline, demographic and biological characteristics were mostly similar by cocaine use (Table 2). However, there was significant difference in CD4 among cocaine users and non-users with cocaine users having lower CD4 cell counts than non-users, p=0.046. Moreover, oxidized glutathione (GSSG) at baseline was higher among cocaine users and was approaching significance, with a p=0.082.

The participants showed 68.4% adherence to the supplement regimen, where intervention group was 75% adherent, while the placebo group was 63% adherent to the pills (Table 3). The intent-to-treat analyses revealed that adherence with

supplementation significantly decreased SB/ALS + oxidized purines after three-month supplementation p=0.001 (Table 4). Pill adherence was significantly associated with measures of oxidatively DNA damage, SB/ALS + oxidized purines, oxidized purines only, and SB/ALS (p=0.001, p=0.020, p=0.029, respectively).

In a linear regression model, when looking on the effect of supplementation on the change of hs-CRP, after three-month supplementation we observed significant associations with gender p=0.011 and body fat % p=0.022, when adjusting for age and BMI (Table 5). In the final model, supplementation was significantly associated with gender p=0.005 and Fat % p=0.015, without adjusting for other variables. Among the intervention group, change in hs-CRP was significantly associated with GSSG p=0.019 and with BMI p=0.004 from baseline to end of clinical trial (Table 8). In Table 7, we observed that cocaine use was approaching significance in the change of BMI p=0.06 and SB/ALS p=0.054 after supplementation.

Discussion

In our double-blinded randomized clinical trial, levels of hs-CRP significantly decreased after three-month of supplementation with NAC and Gly. This is in agreement with findings from Sekhar et al,[10] as they found that supplementation with NAC and Gly significantly decreased levels of inflammation in PLWH. Another similarity was that we observed decrease in body fat % after supplementation. An important difference from Sekhar et al.[10] was that we included both genders. However, our findings also indicated that there was gender differences in improving hs-CRP after three-month supplementation among males, similar to the findings from Reingold et al.[4] In another RCT, Alfonso et al. [12] supplemented NAC to people exposed to asbestos and, different from our findings, found no evidence that

supplementation reduced biomarkers of inflammation.[13] These differences may be explained by the differences in the population study, health conditions, sample size and supplementation doses.

Compliance and adherence to the supplement in this small cohort was a limitation of the study. We experienced high dropout rate, which was not expected, but probably related to the frequent visits and low incentive compared with the incentives offered by the parent grant, the MASH cohort. The baseline visit coincided with the visit to the parent grant, but for the subsequent monthly visits we were offering only $10 for pill counts, dispensation and blood draw. However, our study led to significant findings on the relationship between adherence to the supplements and oxidative DNA damage. In the intervention group 75% of the participants were adherent to their supplements, and we found that those adherent with NAC and Gly supplementation experienced reduction in inflammation. We observed significant hs-CRP reduction after NAC and Glu supplementation, with significant decrease in body fat % and BMI. In our study, CRP decreased among males, similar to the findings of Reingold et al.[4] The decrease in body fat % among PLWH may have been associated with elevated hs-CRP levels.[10]

In the intervention group we observed that supplementation for three months reduced GSSG, which was significantly associated with the reduction in the biomarker of inflammation.

Cocaine use appears to have a significant effect on biological HIV marker. In this clinical trial cocaine users represented 50% of the population in both groups. Also, regardless of supplementation, CD4 cell count was significantly lower among the cocaine users (p=0.045) at baseline. This may indicate that cocaine users, despite

having undetectable viral load, their CD4 cell count has not recuperated to the levels of non-users. Another observation among cocaine users is that oxidized glutathione (GSSG) was higher with an average of 78.67 μM among users compared 55.13 μM to non-users, p=0.082.

After three-month supplementation, we observed that BMI and SB/ALS changed among cocaine users. BMI increased with an average of 0.47 kg/m^2 among cocaine users compared to an average decrease 0.5 kg/m^2 in non-cocaine user. We observed a decrease in SB/ALS in both groups, however, the average decrease in the cocaine users was -11.3 % tail DNA compared to -14.4 % tail DNA in the cocaine non-users, although did not reach significance.

Strengths and Limitations

The major limitation of our study remains small sample size for estimating our experimental aims and high dropout rate. Our strength is that this is a double blinded randomized clinical trial with a control group, which is the ideal design to determine the effects of an intervention, that the length of the study was longer than other relevant studies in the literature, and adequate to observe significant findings in the outcome variables.

In conclusion, our findings show that NAC and Gly supplementation is effective in reducing elevated levels of inflammation after three-month. Furthermore, it decreased body fat %. Cocaine users experienced more oxidative damage and higher inflammation than cocaine nonusers.

Table 1: Baseline Characteristics of study participants by groups

Variable	Intervention N=15	Placebo N=15	*p*-value
Age (years)	53.4±6.9	55.8±5.9	0.29
Gender (M) (%, *n*)	84 (12)	53 (8)	0.13
BMI (kg/m^2)	26.92±2.3	25.35±3.7	0.173
Ethnicity(%, *n*)			
African Americans Hispanic White	66.7 (10) 13.3 (2) 20 (3)	80 (12) 13.3 (2) 6.7 (1)	0.322
CD4+	546±298	577±230	.759
Viral Load	0.15±0.55	0.066±0.25	.590
TNF-α	9.34±11.07	8.125±4.7	0.707
Fat percentage	25.91±5.8	30.12±7.7	.104
CRP	8.15±16.2	6.02±7.197	.648
DNA damage (-hOGG1)	4.6375 ±1.95	6.06±2.8	.118
DNA damage (+hOGG1)	16.8±2.9	17.89 ±4	.407
Total Glutathione (GSH)	962.6±361	902.98±237	.597
Oxidized Glutathione (GSSG)	66.9±37.36	66.8995±38.09	.999

Data are means ± SD. *P* is considered significant at < 0.05

Table 2: Baseline Characteristics of study participants by cocaine use

Variable	User N=15	Non-users N=15	*p*-value
Age (years)	54.4±5.6	55±7.09	0.733
Gender (M) (%, *n*)	73.3 %(11)	60%(9)	0.456
BMI (kg/m^2)	26.92±2.3	26.31 ±3.03	0.347
Ethnicity(%, *n*)			
African Americans Hispanic White	66.7 (10) 20 (3) 13.3 (2)	80 (12) 6.7 (1) 13.3 (2)	0.623
CD4+	465±208	659±2275	0.046*
Viral Load	0.214±0.57	0.0±0.	0.178
TNF-α	7±5.24	10±10.32	0.325
Fat percentage	28.91±6.3	27.13±6.318	0.500
CRP	6.90±6.9	7.27±16.51	0.937
SB/ALS	5.31 ±2.43	5.38±2.6	0.948
SB/ALS+ oxidized purines	16.98±3.3	17.72 ±3.69	0.564
Total Glutathione (GSH)	998.5±261.7	867±332.7	0.239
Oxidized Glutathione (GSSG)	78.67±39.57	55.13±31.36	0.082

Data are means ± SD.
**P* is considered significant at < 0.05

Table 3: Percentage of pill adherence according to the pill count and adherence questionnaire in both groups

Group	Adherence N= 13 (68.4%)	Non-adherence N=6 (31.6%)
Control	63.6% N=7	36.3% N=4
Intervention	75% N=6	25% N=2

Table 4: Linear Regression Models (1,2 and 3) on the effect of the Intervention and Pill Adherence on Changes in oxidatively DNA damage from Baseline to 12-week visit.

Variable Model 1	B	95% CI	β	t	p-value
Intervention	-3.652	[-6.131, -1.172]	-.472	-3.122	0.007*
Pill adherence	-4.860	[-7.494, -2.226]	-0.591	-3.912	0.001*

Dependent Variable: Difference SB/ALS + oxidized purines from baseline to Visit 4
*Statistically significant < 0.05

Variable Model 2	B	95% CI	β	t	p-value
Intervention	-2.369	[-5.789, 1.050]	-0.291	-1.469	0.161
Pill adherence	-4.413	[-8.045,-0.781]	-0.510	-2.576	0.020*

Dependent Variable: Oxidized purines only from baseline to Visit 4
*Statistically significant < 0.05

Variable Model 3	B	95% CI	β	t	p-value
Intervention	-1.863	[-4.952, 1.225]	-0.261	-1.279	0.219
Pill adherence	-3.721	[-7.001, -0.440]	-0.492	-2.404	0.029*

Dependent Variable: Difference SB/ALS
*Statistically significant < 0.05

Table 5: Linear Regression Model on the effect of supplementation on inflammation adjusted for gender, age, BMI, and Fat % on changes in CRP from baseline to end of study.

Variable	B	95% CI	β	t	p-value
Intervention	1.984	[-8.216, 12.185]	.088	.420	.681
Gender	16.592	[4.462, 28.72]	0.654	2.955	0.011*
Age	0.346	[-0.452, 1.143]	.193	.936	.366
Baseline BMI	-0.352	[-1.993. 1.288]	-.096	-.464	.650
Baseline Fat %	0.827	[0.142, 1.153]	.581	2.608	.022*

Dependent Variable: change in CRP

*Statistically significant < 0.05

Table 6: Linear Regression Model on the effect of supplementation, gender and Fat % on changes on CRP from baseline to end of study.

Variable	B	95% CI	β	t	p-value
Intervention	3.346	[-6.268, 12.961]	0.148	0.742	0.470
Gender	17.717	[6.109, 29.325]	0.698	3.253	0.005
Baseline Fat %	0.846	[0.187, 1.506]	0.595	2.735	0.015

Dependent Variable: change in CRP

*Statistically significant < 0.05

Table 7: Change in BMI and SB/ALS from baseline to week 12 in cocaine users compared with non-users.

Variable	Cocaine users n=9	Cocaine non-users n=10	p-value
Change in BMI	0.47+1.42	-0.5400±0.685	0.060
Change in SB/ALS	-11.3147±3.475	-14.4734±3.186	0.054

Data are means ± SD. *P* is considered significant at < 0.05

Table 8: Linear Regression Model on the effect of supplementation, GSSG, BMI, and age on changes in CRP from baseline to end of study among the intervention group.

Variable	B	95% CI	β	t	p-value
GSSG	-2.659	[-4.597, -0.721]	-0.732	-3.809	0.019*
Baseline BMI	-5.762	[-11.282, -0.241]	-0.791	-2.898	0.044*
Age	-1.040	[-2.581, 0.502]	-0.511	-1.873	0.134

Dependent Variable: change in CRP

*Statistically significant < 0.05

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Chapter VII: FUTURE RESEARCH

Our findings from this double-blinded randomized clinical trial indicate that NAC and glycine supplementation showed promising results. Supplementation was effective in reducing oxidative DNA damage. This is the first study to assess the effect of oral NAC and glycine on oxidative DNA damage using the comet assay among PLWH. The sample size was small and the dropout rate was high. A greater understanding is needed with regards of the role of NAC and glycine separately on oxidative DNA damage. To observe the effect of supplementation on GSH and GSSG a clinical trial with a higher dosage may be effective in significantly reducing levels of GSH.

Further research is needed to be conducted with a higher supplementation does, larger sample size, a factorial design, and to consider measuring levels of cysteine and glycine pre and post supplementation.

FIU IRB Approval:	06/27/2018
FIU IRB Expiration:	06/27/2019
FIU IRB Number:	IRB-17-0302

FLORIDA INTERNATIONAL UNIVERSITY

ADULT CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Three-month nutritional supplementation to reduce oxidative stress in people living with HIV (PLWH)

PURPOSE OF THE STUDY

You are being asked to be in a research study. The purpose of this study is to assess the effect of nutritional supplementation with a combination of the antioxidants N-Acetyl Cysteine (1,000 mg/day) and glycine (1000 mg /day) or a placebo on markers of oxidative stress and inflammation among PLWH.

NUMBER OF STUDY PARTICIPANTS

If you decide to be in this study, you will be one of 70 people screened for this research study.

DURATION OF THE STUDY

Your participation will require participation for 3 months and you will be asked to visit the clinic 4 times for 3 months: at baseline, beginning of the second and third month, and at the end of the third month. You will be placed at random (like the flip of a coin) in either the control (comparison) group or the intervention group. You will be required to come to the research clinic 4 times: once for the screening, baseline visit and first dispensation, and 3 more visits for follow-up. Your first visit will either coincide with your MASH clinic visit as part of the MASH study or a separate baseline visit. Visits 2, 3, and 4 are additional to the one in the MASH cohort study. In each visit, the following activities will take place:

Visit 1: Recruitment, consenting, screening, baseline and dispensations (45 minutes for questionnaires, urine sample and finger prick or blood-draw)
Visit 2: Beginning of the second month, questionnaire, urine sample, finger prick or blood-draw, adverse events questionnaire and pill count.
Visit 3: Beginning of the second month, questionnaire, urine sample, finger prick or blood-draw, adverse events questionnaire and pill count.
Visit 4: End of the third month, questionnaire, urine sample, finger prick, blood drawing, adverse events questionnaire and pill count

PROCEDURES

If you agree to be in the study, you will be placed in either the control or intervention group at random (like the toss of a coin). For both groups you will be asked to do the following:

1. When you come for your appointment for the MASH study, you will be asked if you would like to participate in this study, explain the study and consent you if you are willing to participate. We will ask you if you allow us to access your medical and laboratory records maintained in the MASH cohort under your research ID at the end of this consent form.

2. With your permission, any remaining frozen blood will be used for future research.
3. The baseline visit for this study will occur after the screening and consenting, which might be at the same time you come to the MASH study visit. At the baseline visit, and at the beginning of the second and third month in the study, your visits will include finger prick or blood-draw depending on participant preference and blood flow, pill dispensation, urine sample for measures of oxidative stress, and anthropometric measurements (weight, waist circumference). You will be asked to bring your pill bottle with you to count the leftover pills. These visits will last approximately 45 minutes.
4. At the end of this study, the end of the third month, you will come for weight, waist circumference, 24-hour recall, blood drawing and a urine sample. You will be asked to bring your pill bottle with you. This visit should take 30 min.
5. You will be contacted once weekly by telephone to remind you about your pills, follow-up with your health, and ask about side effects.

RISKS AND/OR DISCOMFORTS

The following risks may be associated with your participation in this study: Blood drawing include: discomfort with needle, a possibility of bruising, and fainting. Adverse effects from the supplements could be: upset stomach, fatigue, or skin rash, although these are rare occurrences.
For women only: If you are pregnant or intending to get pregnant, you will be excluded from the study because you will need prenatal vitamins. If you become pregnant during the study you will be terminated and recommended to obtain adequate supplementation.

BENEFITS
The following benefits may be associated with your participation in this study:

1. Nutrition consultations.
2. Opportunity to find out more about yourself and keep track of your health.
3. Chance to contribute to the field of science and HIV treatment.
4. Those in the intervention group may benefit from receiving a nutritional supplement that is an antioxidant.

INCIDENTAL FINDINGS
Although the primary aim of this Nutritional Supplementation Clinical Trial is to improve the antioxidant defense system and prevent and/or delay the onset of inflammation, if you develop any symptoms related to your supplementation during the study, we will refer you to your primary care physician with your permission, stop the supplementation immediately, and unblind your supplements.

ALTERNATIVES
You may receive nutritional supplementation similar to the one provided in this study without prescription, as this combination is considered an over-the-counter antioxidant. Any significant new findings developed during the course of the research which may relate to your willingness to continue your participation will be provided to you.

CONFIDENTIALITY

The records of this study will be kept private and will be protected to the fullest extent provided by law. The MASH cohort has a Certificate of Confidentiality that will be extended to this study. To help us protect your privacy, we have a Certificate of Confidentiality from the National Institutes of Health (NIH). With this Certificate, we can't be forced by a court order or subpoena to disclose information that could identify you in any civil, criminal, administrative, legislative or other proceedings. There are circumstances where the Certificate doesn't protect against disclosure of your personally identifiable information:

- when the US government is inspecting or evaluating federally-funded studies
- when information must be disclosed to meet FDA requirements
- if you give someone written permission to receive research information or you voluntarily disclose your study information
- if the researcher reports that you threatened to harm yourself or others
- in cases of child abuse reported by the researcher
- if the investigator reports cases of contagious disease (such as HIV) to the state

In any sort of report, we might publish, we will not include any information that will make it possible to identify a subject. Research records will be stored securely and only the research team will have access to the records. However, your records may be reviewed for audit purposes by authorized University or other agents who will be bound by the same provisions of confidentiality.

The U.S. Department of Health and Human Services (DHHS) may request to review and obtain copies of your records. The Food and Drug Administration (FDA) may request to review and obtain copies of your records.

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

COMPENSATION & COSTS

At each of the four study visits you will receive a payment of $10 for a total of $40. You will not be responsible for any costs to participate in this study.

MEDICAL TREATMENT

Routinely, FIU, its agents, or its employees do not compensate for or provide free care for human subjects in the event that any injury results from participation in a research project. If you become ill or injured as a direct result of participating in this study, contact your regular medical provider. If you have insurance, your insurance company may or may not pay for these costs. If you do not have insurance, or if your insurance company refuses to pay, you will be billed. Funds to compensate for pain, expenses, lost wages and other damages caused by injury are not routinely available.

STORED BLOOD

With your consent, some of your blood that is left over, after all necessary study testing is done, will be stored. Your blood will not be identified by your name or by other personal information but would instead be identified by a number that could later be linked to other information that was collected as part of the study. This blood will be used for future HIV and antioxidants related research that will be approved by the Florida International University Institutional Review Board and the United States National Institutes of Health, which is sponsoring this study. Please decide if you agree to have some of your blood stored for this reason; you will indicate your decision on the last page of this consent

form. You may withdraw your consent for the use of specimens at any time. If you decide in the future that you do not want your leftover blood to be used for research, study personnel will notify the Laboratory Manager, who will have the specimens discarded.

RIGHT TO DECLINE OR WITHDRAW
Your participation in this study is voluntary. You are free to participate in the study or withdraw your consent at any time during the study. Your withdrawal or lack of participation will not affect any benefits to which you are otherwise entitled. The investigator reserves the right to remove you without your consent at such time that they feel it is in the best interest.

RESEARCHER CONTACT INFORMATION
If you have any questions about the purpose, procedures, or any other issues relating to this research study you may contact Adriana Campa at Florida International University AHC 5- 328, 305-348-2871, campaa@fiu.edu.

IRB CONTACT INFORMATION
If you would like to talk with someone about your rights of being a subject in this research study or about ethical issues with this research study, you may contact the FIU Office of Research Integrity by phone at 305-348-2494 or by email at ori@fiu.edu.

PARTICIPANT AGREEMENT
I have read the information in this consent form and agree to participate in this study. I have had a chance to ask any questions I have about this study, and they have been answered for me. I understand that I will be given a copy of this form for my records.

☐ I consent to the release and sharing of medical and laboratory information between the records of the MASH cohort and this study. _____
Initials

☐ Check here if you agree to allow extra blood to be stored for future HIV related research.
☐ Check here if you DO NOT agree to allow extra blood to be stored for future HIV related research.

_____ _____
Signature of Participant Date

_____
Printed Name of Participant

_____ _____
Signature of Person Obtaining Consent Date

_____
Printed Name of Person Obtaining Consent

VITA

ALHANOOF AL-OHALY

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PUBLICATIONS AND PRESENTATIONS

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