THE EFFECT OF NICOTINAMIDE RIBOSIDE ON LDL-CHOLESTEROL INDUCED T-CELL DYSFUNCTION

by

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ABSTRACT

Aging is associated with chronic low-grade inflammation ("inflammaging") and is a significant risk factor for multiple chronic diseases, including cardiovascular disease, Alzheimer's disease, and cancer. Inflammaging is caused by the deterioration of the innate and adaptive immune systems, often accompanied by immunosenescence. T-lymphocytes (T-cells), primarily known for their role in the adaptive immune system responding to foreign antigens, are increasingly recognized as contributors to inflammaging via immunosenescence and impaired mitochondrial function. Age-related declines of cellular nicotinamide adenine dinucleotide (NAD+) levels may be the trigger of immunosenescence and mitochondrial dysfunction. However, the mechanisms leading to T-cell mediated inflammaging and mitochondrial dysfunction are not fully known. One mechanism which might contribute to age-related T-cell dysfunction is an increase in endogenous low-density lipoprotein cholesterol (LDL-C) in blood plasma. Increased endogenous LDL-C occurs with aging and has been linked, cross-sectionally, to mitochondrial damage and dysfunction, reduction of ATP synthesis, and increased reactive oxygen species. Subsequently, this damage can lead to various cellular consequences, rapid aging, and disease onset.

Supplementation with nicotinamide riboside (NR), a precursor to nicotinamide adenine dinucleotide (NAD+), might be a novel therapeutic to protect T-cells from the deleterious effects of high LDL-C. In addition to its role as a regulator of cellular reoxidation-reduction reactions, NAD+ is a critical co-substrate for several energy-

sensing and stress-resistance enzymes. These enzymes are referred to as “NAD+-consuming enzymes” and include the silent mating type information regulation of two homologs (sirtuins; SIRTs), poly adenosine diphosphate (ADP) ribose polymerases (PARPs), cyclic ADP (cADP)-ribose synthases, and CD38/156 ectoenzymes. These enzymes contribute to a multitude of homeostatic processes including the maintenance of biological stress resistance, DNA damage repair, and the regulation of immune cell function. Supplementation with NR has been shown to increase NAD+ bioavailability. NAD+ has emerged as a vital and intriguing cofactor for maintaining mitochondrial fitness by up-regulating enzymes that repair mitochondrial DNA. However, whether NAD+ can protect T-cell mitochondria from immunosenescence is unknown. This study aimed to investigate the efficacy of exogenous NAD+ supplementation in protecting young T-cells from the effects of hypercholesterolemic conditions associated with aging. I hypothesized that treatment with LDL-C would impair T-cell mitochondrial respiration and induce T-cell inflammation and that NR would exert a protective effect on T-cells, preserving mitochondrial respiration and reducing the inflammatory response.

Seven adults (6 female/ 1 male) between the ages of 22 and 26 participated in this study. Blood samples were collected in EDTA coated vacutainer tubes from all 7 participants. Peripheral Blood Mononuclear Cells (PBMCs), isolated from whole blood, were further isolated into a pan T-cell sample. The isolated T-cells were later treated and used for the Seahorse XF Analyzer. The supernatant was isolated and frozen to use for the quantification of cytokines. Pan T-cells were treated with high

(4.9 mMol/L) physiologic concentrations of LDL-C and co-incubated with high LDL-C and NR compared to control (Serum-free media, 0 mMol/L). T-cell mitochondrial function and inflammatory cytokine production were assessed by measuring mitochondrial respiration using an extracellular flux analyzer (Seahorse XFe96 Analyzer) and multi-plex protein quantification (Luminex Magpix), respectively. Concentrations of pro-and anti-inflammatory cytokines, specifically interleukin-6 (IL-6) and interleukin-10 (IL-10), were measured across the three treatment groups of the pan T-cell samples. Proinflammatory cytokines up-regulate the production of radical oxygen species (ROS) and anti-inflammatory cytokines reduce the production of ROS.

Contrary to our hypothesis, LDL-C non-significantly augmented T-cell mitochondrial respiration during maximal oxygen consumption rate (OCR), while it non-significantly decreased basal OCR and ATP-linked OCR. Further, the addition of NR to LDL-C treated samples exacerbated mitochondrial respiration in all accounts. Anti-inflammatory cytokine production showed a non-significant decrease with the LDL-C and LDL-C+NR treatments. Proinflammatory cytokine production displayed a non-significant increase within the LDL-C and LDL-C+NR treatment groups.

The additional NR treatment to LDL-C treated T-cells significantly decreased the basal, maximum, and ATP-linked OCR, but it is unclear if LDL-C treatment alone had a significant effect on T-cell mitochondrial dysfunction. Cytokine production was not significantly affected with either treatment at the current sample size and may require more samples to clarify a relationship.

Chapter 1

INTRODUCTION

1.1 LOW-DENSITY LIPOPROTEIN CHOLESTEROL AND ITS RELATION TO MITOCHONDRIAL FUNCTION IN ADAPTIVE IMMUNE CELLS

Advancing age is a significant risk factor for the development of cardiovascular disease (CVD), which is the leading cause of mortality in the United States (Whelton et al., 2017). Several risk factors for Alzheimer's Disease overlap with CVD including hypertension and high endogenous low-density lipoprotein-cholesterol (LDL-C) concentrations (Stampher, 2016). The adaptive immune system, specifically T-lymphocytes (T-cells), plays a vital role in the protection against foreign substances and pathogens by producing proinflammatory cytokines that defend against them. With aging, T-cells become dysfunctional and may lead to chronic inflammation and further underlying disease development. High LDL-C has been potentially implicated in T-cell mitochondrial dysfunction (DeConne et Al., 2020) and it is important to discover and understand the factors that may cause this fault in the human immune system. T-cell dysfunction has been implicated in the development of cardiovascular diseases (CVD); however, the specific mechanisms by which T-cell-mediated inflammation modulates cardiovascular function in humans are not entirely known. Aging is associated with chronic low-grade inflammation, otherwise referred to as “inflammaging,” and is caused by age-related deterioration of the innate and adaptive immune systems (Aw et al., 2007). Preliminary studies lead us to suspect that impaired T-cell mitochondrial respiration may result in dysfunctional T-cells that increase bodily concentrations of proinflammatory cytokines and ROS, resulting in oxidative stress and physiological complications (Ungvari et al., 2018). Mitochondria play an anabolic role in generating essential building blocks for macromolecules and

producing ATP. They have emerged as a signaling platform that coordinates T-cell activation and differentiation (Bantung et al., 2017). These signals depend on mitochondrial stress status, and they mediate mitochondrial ROS production. In T-cells, the balance of glycolysis and oxidative phosphorylation (OXPHOS) influences the proinflammatory phenotype of the cells; a shift away from the TCA cycle and toward glycolysis may make T-cells more proinflammatory (Desdín-Micó et al., 2018).

1.2 NICOTINAMIDE RIBOSIDE, A PRECURSOR TO NAD+ AND POSSIBLE TREATMENT TO PROTECT AGAINST MITOCHONDRIAL ANTAGONISTS

NAD+ (nicotinamide adenine dinucleotide) is an electron-accepting molecule used in the first step/complex of the mitochondrial electron transport chain that produces ATP, a vital energy resource to the body. The cellular concentration of NAD+ steadily decreases with age (Shultz & Sinclair et al., 2016). The significant decrease is caused by a NADase, CD38; the CD38 levels rise with age (Camacho-Periera et al., 2016). CD38, a membrane-bound NADase, hydrolyzes NAD+ to nicotinamide and cyclic (ADP-ribose) (Chini et al., 2018). The levels of CD38 protein levels steadily increase with age and effectively decrease the bioavailability of NAD+ (Schultz and Sinclair, 2016). In mouse models, CD38 knock-out mice exhibited augmented SIRT-3 activity, increased mitochondrial function, a longer half-life for nicotinamide riboside (NR), and protection from NAD+ decline. Many of these aging effects are mediated by the decline of mitochondrial SIRT-3, which can be linked back to CD38 levels. Loss of mitochondrial SIRT3 activity is a cause of age-related metabolic decline (Camacho-Pereira et al., 2016). In this regard, the loss of intracellular NAD+ results in oxidative stress, DNA damage, and altered

mitochondrial function by reducing the activity of NAD+-consuming sirtuin 1-7 (SIRT) enzymes as well as important DNA repair mechanisms (Massudi et al., 2012). Increasing intracellular NAD+ can promote an array of positive health benefits, including augmenting mitochondrial function (Covarrubias et al., 2021).

There are several precursors to NAD+ that can be administered exogenously including niacin (nicotinic acid (NA) and nicotinamide), as well as NR and nicotinamide mononucleotide (NMN). In a landmark study by Gomes et al., 2013, a mouse model was treated with NMN and their findings indicated that mitochondria are regulated by nuclear NAD+ and that the impairment of oxidative phosphorylation OXPHOS function during aging may occur through the depletion of nuclear NAD+ (Gomes et al., 2013). NAD+ precursors may also play a role in treating high cholesterol. NA has been shown to effectively treat hypercholesterolemia when administered in large doses (Garten et al., 2015). However, NR may be a more effective precursor, as it has been shown to raise the amount of NAD+ in human blood by 2.7-fold with a single dose of 1000 mg (Trammell et al., 2016). Furthermore, chronic NR supplementation has been shown to be tolerated, safe, and effective in raising cellular levels of NAD+ metabolism in vivo. An oral NR supplementation was shown to elevate levels of NAD+ in peripheral blood mononuclear cells (PBMCs) by about 60% compared to a placebo (Martens et al., 2018). However, it is unknown whether increasing NAD+ can protect against T-cell mediated inflammation and oxidative stress. In this regard, raising the concentration of NAD+ within T-cells may aid in the recovery of T-cell function. We hypothesize that increasing the concentration of NAD+ in T-cells using NR will protect against LDL-C-induced inflammation.

1.3 SYSTEMIC INFLAMMATION CAUSED BY LDL-CHOLESTEROL AND VASCULAR DYSFUNCTION

It has been shown that circulating LDL-C has been shown to increase with age (Félix-Redondo et al., 2013). LDL-C has become a focus of interest, as it may be a risk factor contributing to age-related T-cell dysfunction, that has the potential to cause chronic low-grade inflammation. There has also been a negative association of LDL-C with measures of mitochondrial respiration, as well as increases in markers of oxidative stress and inflammation in immune cells (peripheral blood mononuclear cells, PBMC), which were obtained from both healthy adults and patients with early-stage heart failure (Li et al. 2015). Our laboratory recently expanded on these findings by showing that an elevation in LDL-C is associated with lower mitochondrial respiration in PBMCs in healthy adults and is independent of age and cardiometabolic risk factors (DeConne et al., 2020). These findings will be further explored in this project to investigate the effects that LDL-C may have on the mitochondrial function of T-cells.

Mitochondria, the location of the electron transport chain (ETC), is a significant producer of superoxide. Superoxide ions occupy a prominent role in vascular diseases. An increase in superoxide contributes to a reduction in nitric oxide (NO) bioactivity, which has been associated with the development of endothelial dysfunction and a higher risk for CVD (Ungvari et al., 2018). Superoxide produces ROS that causes oxidative damage to DNA and other essential molecules. Proliferating T-cells are a substantial source of superoxide due to increased metabolic activity. The bodily defense against superoxide-induced damage is antioxidant enzymes, like superoxide dismutase (Dunn et al. 2015). A moderate amount of ROS is required to activate T-cells and release cytokines (Garcia et al., 2018); nonetheless, too

high of a ROS concentration can lead to T-cell dysfunction and damage (Yarosz and Chang, 2018). LDL-C has been shown to reduce mitochondrial antioxidant defense. It is associated with reducing antioxidant transport across the mitochondrial membrane and with consuming intra-mitochondrial antioxidants. With the decrease and consumption of protective antioxidants, oxidative damage can increase within mitochondria, affecting mitochondrial DNA integrity (Oliveira & Vercesi et al. 2020), reducing mitochondrial function, and induction of inflammation via ROS. Without a defense system, damage to mitochondrial DNA can promote a cycle of chronic inflammation; however, no studies have investigated the direct implications of LDL-C on mitochondria and the cytokine production that ensues due to this.

1.4 PRO-INFLAMMATORY CYTOKINE IL-6 AND ANTI-INFLAMMATORY CYTOKINE IL-10: THEIR RELATION TO VASCULAR DYSFUNCTION.

T-cells produce cytokines that regulate immune response and inflammation. Pro-inflammatory cytokines, like IL-6, produce pro-inflammatory signaling and regulate massive cellular responses, while anti-inflammatory cytokines, like IL-10, are suppressive factors for immune response and inflammation (Dong, 2021). IL-10 has previously been shown to suppress the synthesis of IL-6 (Hempel et al., 1995). IL-6 would ideally be raised and expressed at higher levels if an immune response was initiated, and IL-10 would fall. Conversely, an increase in the anti-inflammatory response by an increase in intracellular concentrations of IL-10 would suppress the synthesis of IL-6 by T-cells. Reactive oxygen species are shown to exert dual action on T-cells. Exposure to high levels of ROS decreases T-cell activation and proliferation, while intermediate levels of ROS are required for their activation (Garcia et al. 2018). When LDL-C is introduced to sampled T-cells, the mitochondrial

electron transport chain (ETC) becomes stressed, leading to increased production of ROS. Increased ROS production activates T-cells, potentially chronically, to release pro-inflammatory cytokines, which can negatively affect cardiovascular function.

1.5 AIMS OF THE CURRENT STUDY

The purpose of this study was to determine the efficacy of NR supplementation in preventing the inflammation induced by high cellular levels of LDL-C on the adaptive immune system, specifically T-cells. Specifically, I examined the effects of NR on modulating the LDL-induced release of pro-inflammatory (IL-6) and anti-inflammatory cytokines (IL-10) from T-cells. In addition, I compared the changes in relative basal and maximum mitochondrial respiration, ATP production, and respiratory efficiency.

1.6 HYPOTHESES

My hypothesis was two-fold 1) Incubating isolated T-cells with exogenous LDL-C will impair mitochondrial respiration and induce an inflammatory response from those T-cells, and 2) Further treating T-cells with an NR treatment will prevent LDL-C induced mitochondrial dysfunction and reduce markers of inflammation.

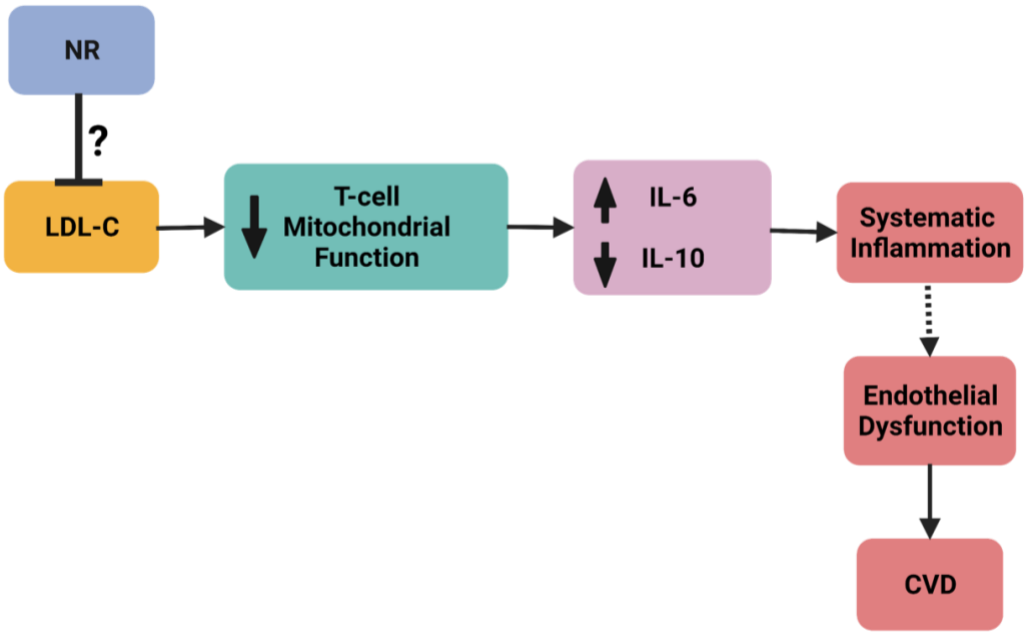

Figure 1. Working hypothesis model. High low-density lipoprotein cholesterol (LDL-C) in blood has been linked to decreased T-cell mitochondrial function. T-cell mitochondrial dysfunction can increase the production of pro-inflammatory cytokines (like IL-6) and inversely, decrease anti-inflammatory cytokines (like IL-10). An increase in pro-inflammatory cytokine production can lead to chronic inflammation, leading to further ailments like endothelial dysfunction, and cardiovascular disease (CVD), Nicotinamide Riboside (NR) is hypothesized to prevent harmful effects of high LDL-C concentrations in blood serum.

Chapter 2

METHODS

2.1 SUBJECT RECRUITMENT

Six female adults and one male adult between the ages of 18 and 29 years old, with a normal circulating LDL-C concentration under 100 mg/dl, were recruited for this study. Subjects were healthy and free of any chronic illnesses and impairments that may interfere with the study results. Subjects were excluded from the study if they were not within the age range, were current smokers, and had a medical history of disease with the coronary artery or peripheral artery, cerebrovascular disease, diabetes, chronic kidney disease, hyperlipidemia, or multiple sclerosis. Additionally, subjects were excluded if they were taking any anti-inflammatory medications, any medication that interfered with endogenous lipid concentrations, or had a body mass index of $>40 kg/m^2$. All procedures were approved by the Institutional Review Board (IRB) at the University of Delaware. Before enrollment, the study rationale, procedures, risks, and benefits were explained to the subjects, and their written, informed consent was obtained. Before their visit, subjects were instructed to fast for at least 12 hours, refrain from exercise and alcohol consumption for 24 hours, and refrain from taking prescription medications for 48 hours.

2.2 SUBJECT SCREENING

A medical history survey was conducted to obtain information regarding any history of chronic illness that may alter the study results. A COVID-19 questionnaire was also administered because COVID-19 can affect a patient's immune system and vasculature.

2.3 PBMC ISOLATION

Whole blood was collected from patients recruited for the pilot LDL-C study conducted in the Neurovascular Aging Lab. The whole blood was isolated into Peripheral Blood Mononuclear Cells (PBMC) with the Miltenyi PBMC isolation Kit. First, the blood was collected into 50 mL conical tubes. Then, it was divided into prepared separation tubes containing 8 ml of separation buffer, 20 µL of RBC removal antibodies, and 180 µL of 0.5 M EDTA stock solution (0.01 M final concentration). The number of tubes prepared corresponded to 1 tube/10 mL of whole blood. Once 10 mL of whole blood was added to each tube, the tubes were gently mixed before centrifugation at 50xg for 3 minutes at room temperature. Once the centrifuge was complete, the top layer of the solution was collected separately, stored in a new 50 mL conical tube, and filled to the top of the tube with a sedimentation buffer. Another centrifuge followed this for 300xg for 5 minutes.

After the centrifugation, the supernatant was discarded. The cell pellets were resuspended in 200 µL of separation buffer. These pellets were then combined to prepare for magnetic labeling and separation. Further, 80 µL of erythrocyte depletion microbeads and 80 µL of granulocyte depletion microbeads (multiplied by the number of tubes) were added for the first whole blood separation. The cell sample was then incubated for 10 minutes.

During the incubation, the cells that were not PBMCs were magnetically labeled. We then used the MACS® magnetic separation columns to run the cell sample through. The column was first washed with 2 mL of separation buffer and then attached to the MACS® magnetic holder. Following the wash, the cell sample was added to the column. The amount of cell suspension should be divided equally among multiple columns. The number of columns used corresponded with the ratio of 1

column/10 mL of whole blood collected. Once calculated, the cell suspension was run through the column and then further washed with 3 mL of separation buffer. This process was repeated with each column. The column trapped all non-PBMC cells and eluted the PBMCs. The eluted cells were combined in one tube. The cell sample was counted on the Invitrogen Cell Countess® II machine (Thermofisher, 2185A18012194). Lastly, the eluted cell sample was spun on 200xg for 10 minutes.

2.4 T-CELL CD4+ AND CD8+ ISOLATION

For the T-cell isolation, 80 µL of separation buffer and 20 µL of CD8+ microbeads per 10^7 PBMCs were added to the isolated PBMC sample. The cells were then incubated for 15 minutes. After incubation, the cells were washed with 2 mL of buffer per 10^7 total PBMCs and centrifuged at 300xg for 8 minutes. The supernatant was discarded, and the pellet was resuspended in 500 µL of separation buffer. A new column was washed as specified by the earlier PBMC separation. The cell sample was run through the column into a 15 mL conical tube labeled "unlabeled cell fraction." The column was washed afterward with 3 ml of separation buffer three times. Then, in another 15 mL conical tube, 5 mL of the separation buffer was added to the column but was taken off the magnetic holder. A plunger included in the MACS® kit plunged out the remaining cells with the magnetic antibody labeling. These cells were the CD8+ cells, with the unlabeled cell fraction containing the CD4+ cells. Both the unlabeled cell fraction and the CD8+ samples were counted.

The cell count was used with the unlabeled cell fraction to add 80 µL of separation buffer and 20 µL of CD4+ microbeads per 10^7 unlabeled cells. The cells were incubated for 15 minutes. After incubation, 2 mL of buffer per 10^7 total cells was added, and the sample was spun on 300xg for 8 minutes. The supernatant was

discarded, and the cell pellet was resuspended in 500 µL of separation buffer. Repetition of previous washing and running of cell suspension through the magnetic column was done for the CD8+ separation. After running the sample and washing the column afterward with 3 ml of separation buffer three times. Then, the CD4+ sample was plunged in an identical process. The CD4+ sample was counted, and the unlabeled cell fraction was discarded.

2.5 CELL CULTURING

After both the CD4+ samples and CD8+ samples were cell counted and centrifuged again on 300xg for 8 minutes. Pre-warmed LDL-C deficient, serum-free AIM-V media was used to suspend the cells. The volume of media warmed corresponded to 1 mL/ 10^6 cells. Once warmed, 1 ml of media was added to each cell sample for every 10^6 cells the sample contained. The suspensions were equally divided into a 48-well cell culture plate. The suspensions were split into 1 mL/well accordingly and stored in the CO_2 incubator for 24 hrs.

2.6 CELL TREATMENT

The incubated cells were added to a treated Seahorse XF 96-well plate at a seeding density of 315,000 cells/well. The CD4+ and CD8+ cells were combined for each plate in a 1:1 ratio, with 150,000 of each type contained in each well. Three treatment groups were used: Control, LDL-C, and LDL-C + NR groups. The control received no LDL-C treatment, while the LDL-C treatments were given 4.9 mMol/L treatment. The 4.9 mMol/L LDL-C concentration for ex vivo treatment was calculated

from 190 mg/dl blood serum concentration, which classifies severely hypercholesterolemic conditions in vivo (Grundy et al., 2019). The LDL-C + NR group treatment received a 4.9 mMol/L LDL-C treatment and a 250 µM NR treatment. The 250 µM NR treatment was determined based on multiple studies that involved in vitro NR treatment of various cell types. One study specifically used a 250 µM NR treatment on hematopoietic stem cells from mice (Zong et al., 2021), and two additional studies involved a 50 µM and 500 µM NR treatment on human brown adipose tissue (Nascimento et al., 2021), and a 100 µM treatment on multiple human cancer cell lines (Wilk et al., 2020). No studies specifically addressed NR ex vivo treatment on T-cells, so 250 µM was used as an intermediate treatment concluded from the three mentioned studies. We also assessed the efficacy of multiple NR treatment concentrations on T-cells in a pilot study. The treatments ranged from 50 µM to 1 mM concentrations, including a 250 µM treatment. The 250 µM NR treatment displayed augmented OCR in that study. NR was obtained from ChromaDex Inc (Irvine, CA) through a material transfer agreement. The samples were incubated for another 24 hr period. After the 24 hr treatment, the Seahorse plate (Agilent, #102416-100) was centrifuged. The supernatant was collected and stored in the -80°C freezer to be analyzed via multi-plex protein bead assay.

2.7 SEAHORSE AND MITOCHONDRIAL STRESS TEST

The mitochondrial (MITO) stress test (Agilent, #103015-100) was performed using the Seahorse XFe96 Analyzer (Agilent, #S7894-10000). The day before the

MITO stress test, the 96-well cell culture miniplate plate (Agilent, #102416-100) was prepped. The wells were coated with 50 µL of a 50 ug/mL working solution of poly-D lysine (Thermofisher, #A3890401) and DPBS (Corning, #21031CV). The coated plate was incubated for one hour in the cell culture hood. After, the poly-D lysine was aspirated off, and three rinses with 100 µL of distilled water took place. A seahorse fluxplate (Agilent, #102416-100) was also prepared, with 200 µL of sterile cell culture water added to each well and stored overnight in the non-CO2 incubator.

The next day, the cells were seeded and treated, and the cell culture miniplate was labeled and seeded with a density of 315,000 cells/well. Each well received 70 µL of the ratioed cell suspension, with 45.5 µL of 12.93 mmol/L (4.9 mMol/L final concentration) of LDL-C per well with the LDL-C treatment, and an additional 4.5 µL of the AIM-V media for each LDL-C well. A 1 mM NR stock solution was used for the LDL-C + NR treatment group, 75.8 µL of the 19.98 mmol/L LDL-C (4.9 mMol/L final concentration) was added to the LDL-C + NR wells, and 50 µL of the 1 mM NR (250 µM final concentration) treatment was then added to the LDL-C + NR treatment wells. An additional 4.2 µL of AIM-V media was added to each of those wells. On the day of the assay, XF RPMI media was heated, and dilutions were made to achieve the concentrations of 10 mM glucose (Agilent,103577-100), 2 mM L-Glutamine (Agilent, 103579-100), and 1 mM sodium pyruvate (Agilent, 103578-100). The Seahorse cell culture miniplate was then spun at 300xg for 8 mins. After the spin, 100 µL media from the wells containing cells were pipetted into a 1.5 ml tube for each well and frozen. An additional 180 µL of XF RPMI assay media was added to the 96-well plate. Before proceeding, cell health, morphology, and purity were viewed under the microscope. The cell culture plate was then placed in the non-CO2 incubator while

prepping the flux plate. The three electron transport chain (ETC) inhibitors were obtained, and stock solutions were made for oligomycin, FCCP, and rotenone/antimycin A. Oligomycin inhibits ATP synthase (complex V). It is injected first in the assay following the basal measurements and it decreases electron flow through the ETC, resulting in a decreased OCR. This decrease in OCR is linked to cellular ATP production. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is an uncoupling agent. It collapses the proton gradient and disrupts the mitochondrial membrane potential. FCCP is the 2nd injection following Oligomycin. It effectively uninhibited electron flow through the ETC, this causes oxygen consumption by complex IV to reach its maximum. The FCCP-stimulated OCR is used to calculate spare respiratory capacity (SRC). SRC is the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure to assess the cell's ability to respond to stress or to an increase in energy demand. The third injection is a mixture of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. This mixture inhibits mitochondrial respiration and allows for the calculation of non-mitochondrial respiration (Chacko et al., 2014).

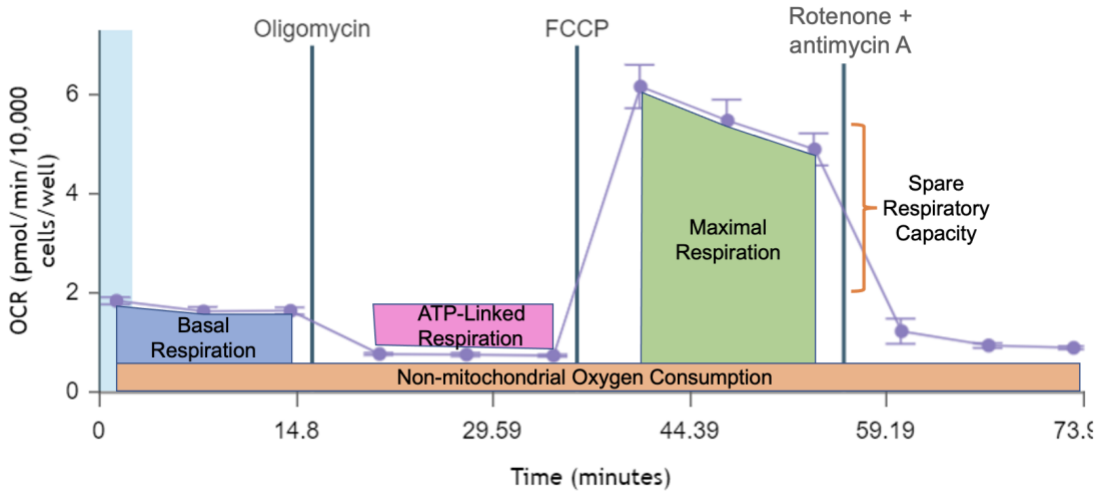

Figure 2. Sample reading of Agilent Seahorse XF Analyzer mitochondrial respiration with inhibitor injections. Data recorded in OCR (pmol/min), data were obtained using one of the study participant's cell samples.

The three inhibitors were loaded into the flux plate with channels labeled A-D. Agilent provided specific volumes to add to each port. The flux plate was then put into the Seahorse XF analyzer and was calibrated for 30 minutes. Following this, the cell culture miniplate was ready to be analyzed and was inserted into the Seahorse analyzer.

2.8 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to compare the means and the spreading of variances of three treatment groups (baseline control, LDL-C, and LDL-C+NR) across multiple mitochondrial respiration assessment measures. An ANOVA was executed when the data analysis was parametric; alternatively, a Friedman ANOVA was used when the data was non-parametric. The basal OCR, SCR OCR, uncoupling coefficient, ATP-linked OCR, and the IL-6 and IL-10 were all run by ANOVA, while maximal OCR was run with a Friedman test as the data was non-parametric or did not fit a normal distribution. The ANOVA and post-hoc statistical significance were set at $P < 0.05$. Post-hoc analyses consisted of Tukey's multiple comparisons test for parametric data, and Dunn's multiple comparisons test for nonparametric data. All analyses were performed with GraphPad Prism 9.1 (GraphPad Software Inc., San Diego, CA, USA).

Chapter 3

RESULTS

3.1 SUBJECT CHARACTERISTICS

Subject Characteristics are summarized in Table 1. Young adults between the ages of 18 and 26 years old were recruited. These adults were generally healthy and free of chronic illness. Their serum LDL-C levels were <100 mg/dl.

Table 1. Participants' demographic and health-related information

Characteristic	Mean ± SD
Sex (Female/Male)	6/1
Age (years)	23.1 ± 1.7
Height (cm)	166.6 ± 7.9
Mass (kg)	64.6 ± 21.4
BMI (kg/m^2)	23.1 ± 6.58
Total Cholesterol (mg/dL)	154.3 ± 16.2
HDL Cholesterol (mg/dL)	58.8 ± 17.4
LDL Cholesterol (mg/dL)	77.5 ± 13.6
Triglycerides (mg/dL)	89.2 ± 81.6
Non-HDL Cholesterol (mg/dL)	95.5 ± 27.3
LDL/HDL ration	1.5 ± 0.9

Values are means ± standard deviation of characteristics for all subjects (n = 7). The total cholesterol, HDL, LDL, triglycerides, non-HDL, and LDL/HDL ratio were tested using the Alere Cholestech LDX® Analyzer, which requires 40 µL of whole blood. \

3.2 SEAHORSE MITOCHONDRIAL STRESS TEST

As reported in Figure 1, mitochondrial respiration was different among the three treatment groups (baseline control, LDL-C, and LDL-C+NR), including Basal OCR (P=0.018, F=1.39), Coupling efficiency (P=0.017, F=2.87), ATP-Linked OCR (P=0.0003, F=12.07), and Maximal OCR (P=0.0084, Friedman=8.86). The SRC OCR data showed a slight difference between the three treatment groups, but this was not statistically significant (P=0.06, F=0.11). Compared to the control, the LDL-C+NR treatment caused a significant decrease in basal OCR (P=0.0446), coupling efficiency (P=0.0476), and ATP-linked OCR (P=0.0025). There was a strongly significant relationship (P<0.01) in maximal respiration with the addition of NR treatment to LDL-C treated cells. The relationships between LDL-C and LDL-C+NR treatments of Basal OCR (P=0.0698) and Coupling efficiency (P=0.0885) were weak trends against the null hypothesis. In addition, the relationships between the baseline control and LDL-C treatment of ATP-Linked OCR (P=0.0693) were weak trends against the null hypothesis, respectively.

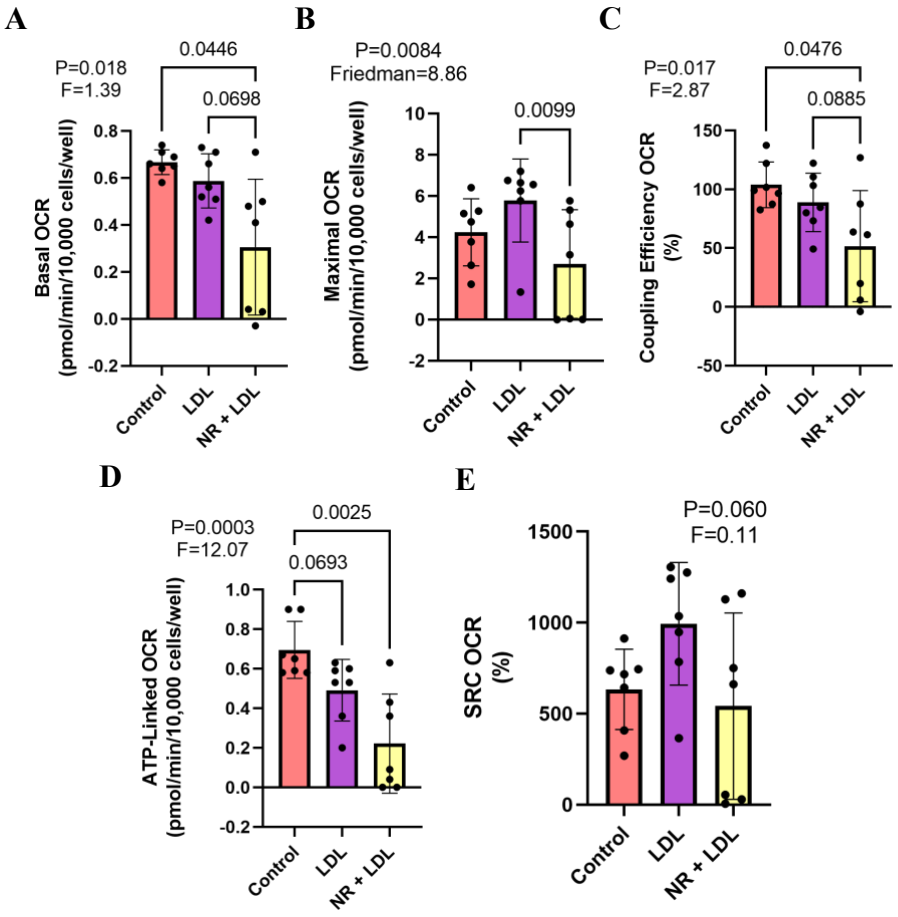

Figure 3. Assessments and markers of mitochondrial functions via Seahorse MITO Stress Test. The difference between mitochondrial Basal OCR (A), Maximal OCR (B), Coupling Efficiency (C), ATP-Linked OCR (D), and SRC OCR (E). α was set at $P < 0.05$.

3.3 CYTOKINE PRODUCTION AMONG TREATMENT GROUPS

To measure the inflammatory effects of three treatment groups, pro-inflammatory IL-6, and anti-inflammatory IL-10 were measured using a multi-plex protein bead assay. There was no significant difference in the IL-6 production between the treatment groups (P=0.29). IL-10 showed no significant difference among treatment groups (P=0.28), but there seemed to be a trend with lower expression in

both LDL-C and NR+LDL-C treated cells compared with control cells. More participant samples may elucidate this relationship.

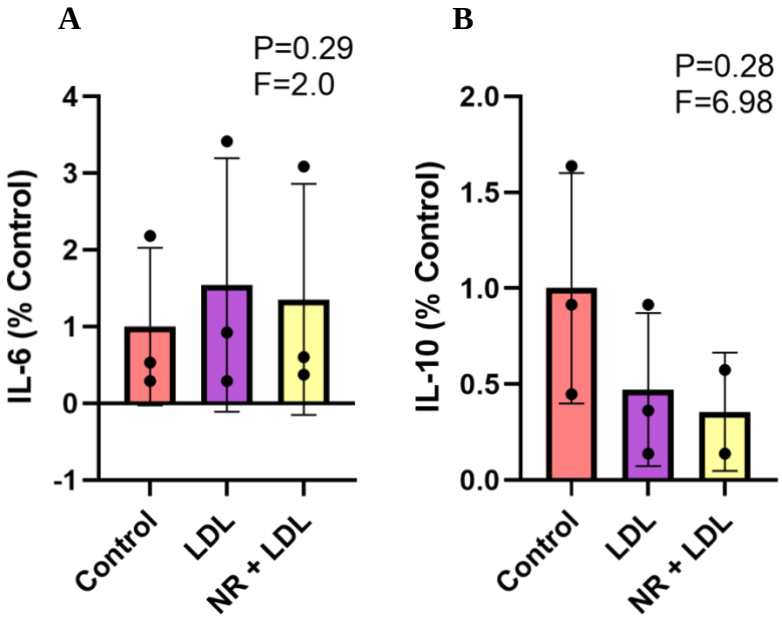

Figure 4. IL-6 and IL-10 Cytokine levels among treatment groups tested with ELISA. Shows the difference between the cytokine concentrations of the supernatant of three patients' T-cell samples, IL-6 (A) and IL-10 (B). α was set at $P < 0.05$.

Chapter 4

DISCUSSION

In this study, we evaluated what effects an LDL-C treatment and an LDL-C+NR combination treatment can have on mitochondrial function and the production of pro- and anti-inflammatory cytokine markers. We attempted to see if the NR treatment could effectively prevent the harmful effect of LDL-C-induced inflammation in attempts to find additional and alternative uses for NR, a molecule that has been a popular subject of research to boost bodily levels of NAD+. NAD+ is used by sirtuin enzymes, a family of enzymes of NAD+ dependent deacetylases, that rely on and deplete NAD+ reserves to execute essential and beneficial cellular functions. Sirtuins are used in the mitochondria to orchestrate their function and adapt to metabolic stresses (Lombard et al., 2011, I. H. Lee, 2019).

We were able to seed, treat, and run Seahorse Mito Stress Tests and multi-plex protein assays to assess mitochondrial function and cytokine production in CD4+ and CD8+ T-cells. Significant relationships and trends displayed the opposite of the proposed hypotheses. Treatment with exogenous LDL-C slightly decreased basal OCR, ATP-linked OCR, and Coupling Efficiency OCR, however, it seemed to slightly augment maximal OCR, none of the treatments with just LDL-C were significant against the control. Furthermore, the addition of NR had the opposite effect on OCR in all mitochondrial measures, displaying significantly decreased OCR against the control. The NR treatment instead of preventing dysfunction exacerbated it. The cytokine production of anti-inflammatory IL-10 decreased, however non-significantly, in both LDL-C and LDL-C+NR treatments. While pro-inflammatory IL-6 saw a non-

significant increase with both treatments. Having a higher participant pool with more sampling may further clarify the relationship that this study sought to explore.

4.1 MITOCHONDRIAL FUNCTION SUFFERED FROM THE ADDITION OF NICOTINAMIDE RIBOSIDE TREATMENT IN COMBINATION WITH LOW-DENSITY LIPOPROTEIN CHOLESTEROL

As indicated by the study results in Figure 2, mitochondrial respiration was worsened by the co-treatment of LDL-C+NR. The impaired mitochondrial function may lead to immune cell dysregulation, which would induce inflammation. Although the supplementation with NR, in theory, would increase NAD+ bioavailability for the use of sirtuins and PARPs, supplementation did not yield these results. These conflicting results require further investigation into the relationship between LDL-C and NR that might impair mitochondrial respiration. In addition, there is a possible difference between in vivo NR supplementation and ex vivo cell culture treatment, which may contribute to the results.

In our study we used a baseline control that treated the cells with an LDL-C deficient, serum-free media: however, it would be appropriate to assume that some amount of cholesterol should be in the media during the experiment in order to simulate in vivo conditions. A media that contained a controlled concentration of LDL-C may have maximized mitochondrial function. The values for OCR recorded during the Seahorse Mito Stress Test may not have been entirely accurate at depicting baseline OCR. The expected control OCR would be higher with an amount of LDL-C that would simulate that of a healthy adult.

This study aimed to analyze the effectiveness of NR supplementation in preventing mitochondrial dysfunction and inflammation linked to LDL-C on immune cells. We hypothesized that incubating isolated T-cells with LDL-C would induce

inflammatory responses from CD4+ and CD8+T-cells. Further, treating T-cells with an NR treatment will prevent LDL-C induced mitochondrial dysfunction and reduce markers of inflammation. The data for this study showed that against a baseline control, the LDL-C-only treatment was not significantly different. However, the baseline control may not have been a wholly accurate representation of the nature of cholesterol state in vivo. The blood serum will always contain some amount of cholesterol at a normal baseline, so only relying on the intracellular reserves of cholesterol for this experiment may have produced misleading results. Nonetheless, the Seahorse results expressed that the NR and LDL-C combination treatment significantly decreased the basal, maximal, and ATP-linked OCR, and OCR coupling efficiency. Unless there are unknown confounding variables present, we have found evidence contradicting the second hypothesis that may lead to further research investigation in the future.

Perhaps the addition of multiple stressors on the cell at one time was enough not to improve the mitochondrial function but hamper it. In addition to sirtuins, PARPS (Poly ADP-ribose polymerases) use NAD+ to produce ADP-ribose molecules. PARPs also respond to DNA breaks and facilitate repair. As DNA damage can accrue, the activation of PARPS increases and effectively lowers the activation of sirtuins due to substrate competition (Mehmel et al., 2020). While the current study's treatment was short-term, the high LDL-C treatment may raise ROS expression enough to severely damage mitochondrial and nuclear DNA and create substrate competition between sirtuins and PARPs. While PARPs are generally good because they repair DNA, a decrease in sirtuin activity is related to the repercussions of aging. Sirtuins are integral to mitochondria function as they are responsible for ATP- homeostasis, ROS

detoxification, DNA repair, and apoptosis suppression (Mehmel et al. 2020). The lack of sirtuin-substrate binding could cause further ramifications of these factors being dysregulated. Though we are increasing NAD+ bioavailability, we may be causing there to be more competition and more strain on the T-cells between essential enzymes. Future studies could incorporate the detection of PARPs (like PARP1) or sirtuins (SIRT3) activity, which are associated with mitochondrial homeostasis, to investigate this competition effect on the T-cell mitochondria.

In T-cell mitochondria, a balance must occur to maintain T-cell homeostasis—mitochondrial ROS act as signaling messengers in various cellular processes, including T-cell activation. While high ROS is associated with cell damage, intermediate ROS levels are associated with survival and activation. When we block OXPHOS, the T-cells move into a proinflammatory state, but when we block glycolysis, we push the T-cells into an anti-inflammatory state. Without a certain amount of OXPHOS, the T-cells will not be able to activate correctly (Desdín-Micó et al., 2018). The NR may have reduced ROS production to a threshold that the T-cells are less able to activate and proliferate, and, further, cannot maintain mitochondrial demand and become dysfunctional.

However, NAD+ bioavailability may not increase significantly when NR is co-treated with LDL-C, as LDL-C may influence the homeostatic balance of NAD+ and its reduced form NADH. NAD+ and NADH are mediators of important and numerous biological processes, including mitochondrial electron transport, oxidative stress, energy metabolism, and aging. The ratio of NAD+ to NADH is an index of cellular reducing potential, and it can be altered under various conditions like high LDL-C. Complex I is the leading consumer of NADH in the mitochondria, which oxidizes it

back into its reduced NAD+ state. Dysfunction of complex I may affect NADH oxidation. In a study on human aortic endothelial cells, treatment with LDL-C significantly reduced the NAD+/NADH ratio, indicating damage or dysfunction to complex I of the ETC (Chowdhury et al., 2010). Further, another study using bovine heart cells suggested that the inhibition of complex I reduce NAD+ levels and is associated with enhanced superoxide production (Kussmaul et al., 2006). Thus, the treatment with LDL-C may prevent the improved bioavailability of NAD+ with NR supplementation and lead to possible ETC dysfunction and increased superoxide production.

4.2 TREATMENT GROUPS DISPLAYED NON-SIGNIFICANT CHANGES IN CYTOKINE PRODUCTION

T-cells produce pro-inflammatory and anti-inflammatory cytokines, which chemically induce tissue damage repair, control cell replication, and apoptosis, and modulate immune reactions (Foster, 2021). IL-6 is a pro-inflammatory cytokine that usually has an inverse relationship to the production of IL-10, an anti-inflammatory cytokine. IL-10 has previously been shown to suppress the synthesis of IL-6 (Hempel et al. 1995). The relation between IL-6 and IL-10 was further explored in this study. They were used as markers of inflammation to assess the hypothesis for NR and LDL-C treatment. IL-6 levels were expected to increase with LDL-C treatment, and IL-10 was suspected to decrease. IL-10 production was lowered non-significantly, and IL-6 was increased but non-significantly in both treatment groups. It is possible that with the IL-6 and IL-10 data, the high variance allowed for inconclusive results. There were three patient samples used from the original seven with the multi-plex assay. With more replicates and patients, there may have been more conclusive results. Although

somewhat displaying the expected inverse relationship, these results display the opposite expectation to what we initially hypothesized. The IL-10 was suspected to rise with the addition of NR to curb the inflammation. However, the opposite occurred: with and without NR, the LDL-C treatment had non-significant decreases in anti-inflammatory IL-10 production and a slight but non-significant increase in IL-6 production. In mouse hepatocyte, AML12 cells, markers of inflammation, including IL-6 and TNF-α, were decreased in NR-treated cells (H.J Lee & Yang 2019). The expected relationship was not seen in this study. We suggest actively searching for variables that may confound the results in the future. Otherwise, many of our patients were in their 20s and generally had healthy BMIs and cholesterol panels. Perhaps our findings reveal a counter indicative use of NR in young healthy adults, where "inflammaging" is not yet a concern. NR supplementation has been shown as a viable treatment to augment the function and DNA integrity of a plethora of dysfunctional cell types in human and mouse models. It may be that NR supplementation in young adults has non-beneficial effects that have not been explored.

Moreover, our findings do not indicate that the NR treatment had the hypothesized effect. We suggest that future studies explore the relationship between NR and more severe risk factors related to cardiovascular diseases. Hypercholesteremic conditions may not be relieved with the addition of an NR supplement into the diet, quite the opposite.

4.3 LIMITATIONS OF STUDY

Some of the significant limitations of this study were the small sample size (n=7) and the uneven distribution of 6 female subjects and 1 male subject. Additionally, there was a lack of an accurate physiological control for LDL-C. The T-

cells were all from healthy adults, and the state of hypercholesteremia was simulated and not a natural condition of the patient in vivo. I compared acute high endogenous LDL-C exposure to a baseline control ex vivo. Still, the effects of chronic exposure or a natural state of high LDL-C cholesterol in vivo were not observed. In addition, the baseline control media of the experiment was not treated with LDL-C because there was no standard value of cholesterol that our lab knew of that would simulate baseline cholesterol in an average adult via exogenous treatment. The cells relied on their stores of LDL-C present in the participant at the time of the blood draw. A future study could incorporate the data from the participant's recorded lipid panel to calculate a participant-specific control treatment. Alternatively, a standard baseline control could use one LDL-C concentration that corresponds to a concentration in the range of healthy cholesterol levels of an adult, but pilot studies are required to test for discrepancies between the two alternatives. We also cannot ensure that the LDL-C treatment would properly penetrate the T-cells ex vivo or that the markers of inflammation were not somewhat lost during the multi-plex protein experiment. Unfortunately, the T-cell supernatant from three patients could only be used, so the cytokine graphs in figure 2. are not representative of all seven patients and lack statistical power.

The replicates for each treatment group were small (n=3) due to the limited space allotted on the seahorse plate and the limited number of T-cells that we could isolate from the participant. Also, many other participants were disqualified, did not show up, or gave too little blood to execute the seahorse experiment, and those participants were excluded from the study.

4.4 CLINICAL RELEVANCE AND FUTURE DIRECTIONS

Investigating immune-inducing factors that can develop and contribute to inflammaging can lead to the discovery of lifestyle and supplemental changes that can prevent chronic inflammation. Chronic inflammation has proven to be a sign and cause of many cardiovascular diseases, cancers, and neurodegenerative diseases. Previous research has shown that LDL-cholesterol is linked with chronic inflammatory processes when retained in the arterial wall (Linton et al., 2019). T-cells recognize LDL-C and cause their activation toward immune response (Gisterå et al., 2018). Treating this inflammation with interventions to reverse the damage is essential in the next step in translational research. NAD+ and its intermediate molecules, like NR, have been shown to enhance insulin sensitivity, restore gene expression from oxidative stress, and regulate inflammatory responses through the activations of sirtuins.

Furthermore, NAD+ levels show a significant drop in many organs during aging (Yoshino et al. 2011), leading to an interest in replenishing NAD+ reserves and investigating its connection to inflammaging. Previous research has shown that reducing risk factors associated with cardiovascular disease is associated with a decreased risk of developing Alzheimer's Disease (Li et al., 2011). Our lab is currently conducting a clinical trial that gives daily doses of NAD+ supplements to adults with mild cognitive impairment (MCI), an early stage of dementia. Our goal is to understand risk factors and identify lifestyle and supplemental changes that can lower the risk of cardiovascular disease that may ultimately lead to the development of MCI and Alzheimer's disease (AD). Our lab suggests early interventions to slow the progression of cognitive impairment, primarily due to risk factors that overlap with CVD.

This project simulated hypercholesterolemic conditions in T-cells ex vivo and attempted to discover any influence that NAD+ had on the T-cell's inflammatory response. It is crucial to classify the safety that NAD+ supplementation will have for people with severe preexisting conditions. Comprehending which pro- and anti-inflammatory cytokines are markers of inflammation due to factors of CVD will help determine identifiable measures for treatment.

Future studies should establish the impacts of LDL-C cholesterol on the inflammatory system; there have seemingly been contradictory findings that slow the profession of immune-based cardiovascular therapy (Gisterå et al., 2018). Additional studies need to clarify how activation of LDL-reactive immune cells may impact cardiovascular function. Further characterization of NAD+ supplementation needs to clarify its proper use, versatility, and how it can interact with certain risk factors. This project produced contradicting outcomes to prior literature and the general pathways that NAD+ contributes. The LDL-C and LDL-C+NR treatments had non-significant effects on cytokine production. A continuation of this project among a panel of simulated CVD risk factors may prove helpful in developing further research to focus on NAD+ supplementation and how it interacts with preexisting disease. A fourth treatment group that looked at the effects of NR treatment alone, may also give a better indication of NR-specific effects on T-cells, which may have been overlooked by this study. In addition, further studying the concentrations of ROS within treated cell samples would allow us to better understand how these risk factors or novel therapies, like NR, could affect the production of inflammatory markers.

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