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The role of neutrophil elastase in the development of obesity related tissue damage

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THE ROLE OF NEUTROPHIL ELASTASE IN THE DEVELOPMENT OF OBESITY RELATED TISSUE DAMAGE

by

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THE ROLE OF NEUTROPHIL ELASTASE IN THE DEVELOPMENT OF OBESITY RELATED TISSUE DAMAGE

SHOAIB KHAN

ABSTRACT

Obesity is increasing worldwide, and the associated health-risks are also on the rise. Eventually, obesity related tissue damage leads to complications such as chronic inflammation, diabetes, cardiovascular disease, and non-alcoholic fatty liver disease. Adipose tissue expansion in obesity triggers specific mechanisms that cause tissue damage. The immune system is especially agitated with excessive fat accumulation, which triggers inflammation and subsequent immune cell infiltration of tissue. Neutrophils are a major immune cell that cause damage in obesity, and the protease neutrophil elastase (NE) is a major neutrophil released factor of tissue damage. The goal of this study is to use tissue extracted from neutrophil elastase knockout (NEKO) mice that have been fed a high-fat high-fructose diet (HFHFD), and compare them to wild-type (WT) mice fed a normal chow diet (NCD), high-fat diet (HFD), and HFHFD to understand the effect of neutrophil elastase damage in obesity. Tissue from aged (NEKO) mice will also be examined to evaluate the role of neutrophils and NE in tissue damage in aging and obesity. Mice in these experimental groups were sacrificed and had their tissue extracted for various staining protocols to discover the extent of tissue damage and immune cell infiltration between mice with and mice without NE.

One experiment had 4 different diets fed to mice. The other experiment had mice aged for 2 years, and mice aged for 3 months and 4 months. Mice from the first experiment were fed for 4 months and separated into 4 groups based on diet, WT-NCD, WT-HFD, WT-HFHFD, and NEKO-HFHFD. Our data indicates that, in comparison with WT-HFHF mice, NEKO-HFHFD mice had less steatosis, fibrosis, immune cell infiltration, and apoptosis within the liver. Neutrophil infiltration into the liver is increased by the HFHFD diet. HFHFD diet also stimulates fibrosis, as indicated by collagen deposition in the liver. Neutrophil accumulation is also associated with the increase of macrophages and CD4 T_h Cells in the liver, particularly in WT mice fed the HFHFD. Interestingly, the liver from NEKO-HFHFD mice had dramatically reduced infiltration of neutrophils, macrophages, and CD4+ T_h cells. Our data suggests that NE is required for HFHFD induced inflammation and fibrosis in the liver.

Mice from the second experiment were split into 3 groups based on age, WT-Young (3 months and 4 months), WT-Old, and NEKO-Old. All groups were fed the same normal chow diet, but WT Old and NE KO Old were both aged to 2 years old. Our data revealed that NE deletion in aged mice reduced fibrosis, elastin fragmentation, calcification, and presence of NE within the aorta.

While part of the mechanism for neutrophil elastase related tissue damage has been explored through this one-year master degree research project, more work is needed to fully understand how NE is stimulated and causes tissue damage. Future work should

examine the potential interaction between neutrophils and other immune cells in obesity and aging.

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A1AT……. Alpha-1 Antitrypsin Inhibitor or Alpha$_1$–Proteinase Inhibitor

AMP…….. Adenosine Monophosphate

AMPK……. AMP Activated Protein Kinase

ATP…… Adenosine Triphosphate

CCL5…….. Chemokine (C-C Motif) Ligand 5

CXCL1……... Chemokine (C-X-C Motif) Ligand 1

CXCL8……... Chemokine (C-X-C Motif) Ligand 8

DGAT2…….. Diacylglycerol Acyl Transferase 2

FFA…….. Free Fatty Acid

G-CSF……... Granulocyte Colony-Stimulating Factor

GM-CSF……... Granulocyte-Macrophage Colony Stimulating Factor

IL-1B…… Interleukin 1 Beta

IL-6……... Interleukin 6

IL-8……... Interleukin 8

IL-17……... Interleukin 17

IL-33……... Interleukin 33

IRS-1…… Insulin Receptor Substrate 1

HFD……. High Fat Diet

HFHFD……..... High Fat High Fructose Diet

LDL…….. Low Density Lipoprotein

3M…….…….. 3 Month

4M…….….. 4 Month

MMP…… Matrix Metalloproteinase

MPO……. Myeloperoxidase

NAFLD…… Non-Alcoholic Fatty Liver Disease

NASH……... Non-Alcoholic Steatohepatitis

NCD……... Normal Chow Diet

NE…….. Neutrophil Elastase

NEKO……..……. Neutrophil Elastase Knockout

ROS…….. Reactive Oxygen Species

TdT……... Terminal Deoxynucleotidyl Transferase

TNFα……..... Tumor Necrosis Factor Alpha

TGFβ…….. Transforming Growth Factor Beta

WAT……..…… White Adipose Tissue

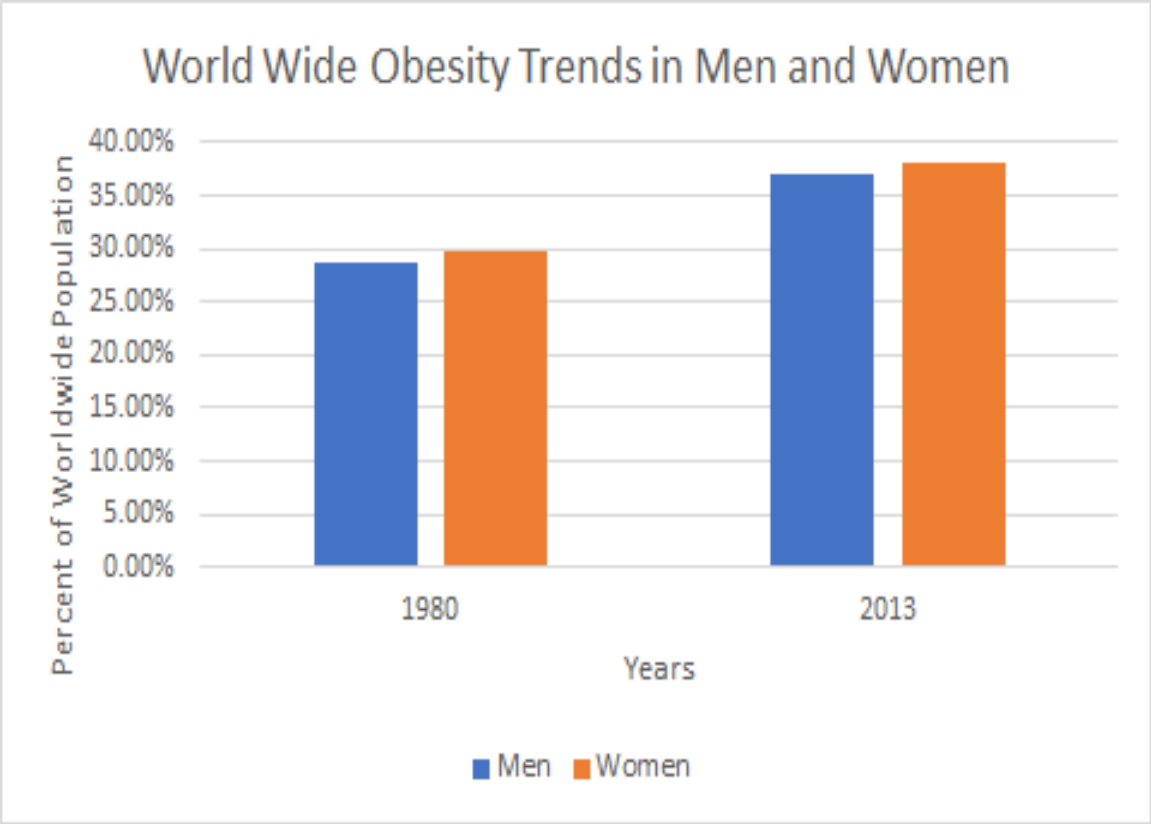
WT……... Wild-Type

INTRODUCTION

Obesity is one of the largest health problems facing the world. By 2030 it is anticipated that around 38% of the world's adult population will be overweight, and 20% will be obese.[1] With upward trends in obesity occurring in developing countries, and morbid obesity still on the rise in many developed western nations, obesity will only become a greater problem in the future.[1, 2, 3] Obesity is a risk factor for many other diseases such as cardiovascular disease, diabetes, and non-alcoholic fatty liver disease (NAFLD).[1] In addition, obese people on average will have a shorter lifespan.[1] Thus, the damage that obesity can cause to the human body is immense.

However, what causes tissue damage in obesity? What factors in obesity act to cause the increased risk of obesity-related diseases such as cardiovascular diseases, diabetes, and fatty liver diseases? An increased fat mass alone has been shown to not be the sole factor in obesity related diseases.[4] In cases of liposuction being used to remove fat, often times no increased metabolic benefits were observed.[4] This shows that other factors beyond a simple increase in fat are causing increased risks of disease in obesity.

Figure 1. Global increase of obesity in men and women[2]



Representing the worldwide trend towards an increase in obesity in both men and women. Adapted from Ng M, Fleming T, Robinson M, et al.[2]

INFLAMMATION IN OBESITY

Inflammation is a process that is normally meant to aid the body. Inflammation's end goal is to restore tissue and organ function to normal and healthy levels.[5] There are two types of inflammation, acute and chronic. Acute inflammation is short-term and is characterized by swelling and leukocyte infiltration to the site, with the purpose of maintaining healing bodily functions.[5] Chronic inflammation is characterized by lymphocyte and macrophage infiltration, blood vessel and connective tissue proliferation,

is long lasting; from months to years, abnormal, and results in damage to the body.[5] Adipose tissue in obesity conditions undergoes chronic inflammation, which is a major issue in obesity.[5] Adipose tissue can produce pro-inflammatory cytokines such as Interleukin-6 (IL-6), Tumor Necrosis Factor (TNFα), and Transforming Growth Factor (TGFβ).[5,6,7,8] The levels of these molecules is also often found to be much higher in the serum of obese patients.[5, 6, 7] When WAT mass increases due to an abundant supply of nutrients, this may cause difficulties in supplying blood to WAT. In turn, this can result in possible hypoxia of WAT, which may lead to WAT necrosis and thus be a possible vector for macrophage infiltration into WAT, and another cause of chronic inflammation in obesity.[5] When WAT mass increases due to hyperplasia and hypertrophy, the inflammatory process is triggered within WAT resident macrophages, which switch from an inflammatory suppressing M2 to inflammatory stimulating M1.[6, 9] An increased amount of lipids within WAT and liver due to obesity also results in the recruitment of additional M1 macrophages which will increase inflammation within WAT and liver.[10] M1 macrophages in the visceral WAT were shown to have increased expression of chemoattractants for neutrophils, such as CXCL1 and CXCL8.[11] The M1 macrophages may be able to stimulate neutrophil recruitment into the liver through venous drainage of visceral WAT into the liver portal system.[11] This drainage may be filled with pro-inflammatory cytokines and neutrophil recruitment factors that can result in the recruitment of neutrophils to the liver and the stimulation of Kupffer cells (liver macrophages).[11] This can result in an increase in inflammation, and may start a continuous cycle leading to chronic inflammation in the liver. Macrophages are also

capable of increasing the lifespan of neutrophils.[12] Through the secretion of granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and tumor necrosis factor alpha (TNFα), neutrophil lifespan is increased.[12] This could also result in chronic inflammation. The increased neutrophil population that results due to obesity is capable of releasing elastase; which can damage insulin receptor substrate 1 (IRS-1) on white adipose tissue and liver tissue, causing insulin resistance and lipogenesis within adipose and liver tissue.[13] Neutrophils can therefore contribute to the buildup of WAT mass, and enhance obesity related inflammation.

NEUTROPHIL BASICS AND FUNCTION IN OBESITY

Neutrophils are an immune system cell with functions in both the innate and humoral immune systems.[14] Neutrophils are a granule secreting cell that aims to fight bacterial and fungal infections, as well as moderate the immune response, such as by inhibiting T-cells.[15] In the case of obesity however, neutrophil function seems to be altered. As mentioned previously, one function of neutrophils in obesity is to aid in the development of chronic inflammation in adipose tissue and liver. In addition to inflammatory macrophages, WAT can also increase neutrophil infiltration due to WAT secreting Interleukin 8 (IL-8), another recruitment factor for neutrophils.[7, 15] Neutrophils may then subsequently release Reactive Oxygen Species (ROS), TNF-α, thromboxane, myeloperoxidase (MPO), matrix metalloproteinase (MMP), and other substances.[15] These released chemicals have the capacity to damage the surrounding adipose tissue, which adds another possible cause for the further recruitment of macrophages, and

subsequent chronic inflammation.[15] This increased inflammation could then play a role in other diseases such as Type II Diabetes or non-alcoholic fatty liver disease.[11,16] TNF-α for example, which is upregulated in obesity related adipose tissue, is a contributor to insulin resistance, and thus Type II Diabetes.[16]

PATHOPHYSIOLOGY OF NASH

Obesity, as mentioned previously, can be a risk factor for nonalcoholic fatty liver disease (NAFLD). NAFLD refers to a collection of various diseases/pathologies of the liver, such as fat deposition, light fibrosis and light inflammation; which can develop into more serious cirrhosis and nonalcoholic steatohepatitis (NASH).[17,18] NASH refers to severe inflammation that results from the liver accumulating a large amount of adipose deposits which can lead to lipotoxicity.[19] Adipose deposition in the liver occurs when there is an excess of adipose tissue within the body, which when broken down for energy leads to an abundant supply of free fatty acids (FFA) in the liver.[19] Dietary sources of high fat and high carbohydrates can also be a source of lipid deposition in the liver. Triglycerides are the most common type of lipid to accumulate in the liver due to the large pool of FFA available.[19] The enzyme Diacylglycerol acyl transferase 2 (DGAT2) is responsible for the final step in transferring FFA onto triglycerides.[19] Triglycerides can then be stored in the liver as lipid droplets.

In order to be diagnosed with NAFLD, around 5% of the liver must have undergone macrovesicular steatosis, where fat deposits have displaced the nucleus of hepatocytes.[20,21] For a diagnosis of NASH, there must be several histopathologic lesions

occurring together; such as steatosis, inflammation (lobular and portal), and injury to hepatocytes; typically in the form of ballooning.[20,21,22,23] Ballooning causes hepatocytes to appear enlarged with a translucent cytoplasm that has unusual borders.[20] Ballooning is also associated with oxidative damage, and microtubule disruption.[20] Lobular inflammation is typically more common than portal, and is one of the first areas to be inflamed in the earlier stages of NASH.[21] Portal inflammation shows up in the later stages of NASH.[21] Continued NASH can be a factor leading to cirrhosis in NAFLD, as the constant inflammation and oxidative stress can result in the activation of hepatic stellate cells, leading to fibrosis and subsequent cirrhosis.[21] When fibrosis starts to occur, it typically starts in zone 3 cells of the liver.[21] Next is periportal fibrosis, where collagen strands envelope periportal hepatocytes and can extend to the surrounding parenchyma.[21] This can progress to a bridging of fibrosis. This bridging may originate from perisinusoidal fibrosis, and form networks of fibers between vascular structures.[21] Bridging fibrosis can be central-central, central-portal, or portal-portal.[21]

Apoptosis, programmed cell death, is more common in hepatocytes in NASH due to the many lesions the liver suffers. Apoptosis can also be a cause of fibrosis, and may encourage additional liver inflammation in NASH.[24] When apoptotic bodies are phagocytized by hepatocyte stellate cells, superoxide and TGFβ production are triggered, leading to increased levels of fibrosis, inflammation, and apoptosis in NASH.[24]

NEUTROPHIL FUNCTION IN NASH

When the liver starts to accumulate large amounts of adipose deposits, neutrophil infiltration can occur, as mentioned previously, due to the release of IL-8 from adipocytes and increased M1 stimulation and recruitment. The infiltration of neutrophils into hepatic tissue in NASH is a major pathological feature in liver damage associated with obesity.[25] A study showed that neutrophils may possibly be used as a measure/marker and predictor of NASH; as a high ratio of neutrophils to lymphocytes was found in patients with NASH and advanced fibrosis.[26] However, there is debate as to whether this method is useful, especially when patients have comorbidities such as diabetes.[27]

One manner by which neutrophils cause liver damage in obesity is through the release of MPO, which will generate compounds that can be cytotoxic, or result in increased fibrogenesis.[28,29] MPO can also induce neutrophil activation, thus starting a vicious cycle of neutrophil activation and damage in the liver.[29] In addition, neutrophils, through MPO also cause increased inflammation.[29] An increased expression of Interleukin 17 (IL-17), a proinflammatory marker, has been noted in neutrophils that have infiltrated the portal tracts of liver in NASH.[30, 31, 32] IL-17 is also produced by Th-17 cells, a type of CD4 + T helper cell.[33] Th-17 cell differentiation is stimulated when in the presence of both TGFβ and IL-6, which occurs with high levels of fat accumulation.[33] The increased IL-17 expression can result in a cascade that leads to increased neutrophil recruitment.[33, 34] Thus, a high level of Th-17 cells in the liver could lead to an increased neutrophil recruitment, and thus be another vector for increased inflammation in NASH.

NEUTROPHIL ELASTASE BASICS AND EFFECTS IN NASH

NE is another neutrophil released factor that is thought to play a role in neutrophil mediated damage in obesity. NE is a serine proteolytic enzyme within neutrophils that is stored in azurophilic granules.[35, 36] NE normally functions to cleave outer membrane proteins on gram-negative bacteria, providing antimicrobial defense.[37] NE shows an increased expression in obesity related inflammation, and is also thought to help promote inflammation.[36, 38, 39] When levels of NE inhibitor α1-antitrypsin (A1AT) are low, there are also an increase in the levels of inflammation in NASH, further showing a relationship between neutrophil elastase and inflammation.[38, 40] High levels of ROS can result in the inactivation of A1AT, thus increasing the availability and activity of NE.[41] NE is also shown to play a role in increasing the number of myeloid cells during high fat conditions.[42] When NE is inhibited, there is a decrease in the expression of pro-inflammatory cytokines, like tumor necrosis factor (TNFα), and in the number of pro-inflammatory cells such as neutrophils.[35, 43] There is also a decrease in fat deposition in the liver when NE is inhibited or knocked out.[38, 43] NE may have another role in increasing the generation of lipotoxic species, such as ceramides.[44] Ceramides are a type of cytotoxic lipid that also possess pro-inflammatory activity, and may increase apoptosis of hepatocytes as a result.[44] Ceramides can be made in the liver by the enzyme serine palmitoyltransferase from serine and palmitoyl-CoA.[44] NE may increase the expression of serine palmitoyltransferase, and possibly other lipotoxic generating enzymes; thus upregulating ceramide production, increasing lipotoxicity in the liver, and increasing liver damage in NASH.[44] NE can also degrade the extracellular matrix and breakdown

cadherins; contributing to additional liver damage as tissue integrity is weakened.[37, 45, 46] High levels of neutrophil elastase are also associated with fibrosis in the liver, and it has the ability to activate pro-fibrotic cytokines like IL-1B and IL-33.[45, 47] Thus, NE can contribute to the development of fibrosis of the liver and eventual cirrhosis.[45]

EFFECTS OF HIGH FRUCTOSE DIET

Within developed nations, increases in sugar intake, especially in combination with a sedentary lifestyle are a cause for concern; as they have come to mirror the increase in metabolic irregularities that come from obesity.[48] Fructose consumption is especially high due to additive sugars, soft drinks, and processed foods that are common to a western diet. Increases in fructose consumption, even short term, has been shown to increase incidences of inflammation and neutrophil infiltration in the liver of mouse models.[49, 50] It has also resulted in an increase in markers such as CD11b, an adhesion molecule that can allow for greater neutrophil tissue infiltration.[48] High levels of fructose consumption causes a rapid depletion of ATP and generation of ROS, resulting in the increased levels of inflammation.[51] Unlike glucose, fructose breakdown mostly occurs in the liver.[51] Thus, its breakdown triggers liver inflammation when in large quantities.

High fructose levels will also generate Methylglyoxal, which can block the interaction of AMP and AMPK.[51, 52] This interaction will normally result in the inhibition of acetyl CoA carboxylase, thus decreasing fatty acid synthesis. Thus, high fructose levels enhance fatty acid synthesis, and will increase fat deposition within the

liver.[51, 52] There is also no feedback mechanism to halt fructose breakdown and prevent ATP depletion like with glucose.[53] This means that high levels of fructose are unregulated and can result in more severe NASH development. High fructose consumption has also been associated with increased levels of liver fibrosis as found in a study done on adult human subjects.[54] The effects of fructose on the progression of NALFD and NASH, especially when examined in conjunction with the enzyme neutrophil elastase are of interest. High fructose levels connect to NASH pathologies like inflammation and neutrophil infiltration into the liver. By further investigating fructose and NE interaction in NASH, more understanding into the mechanisms of NALFD and NASH could be achieved, as well as possible future therapy targets.

SIMILARITIES BETWEEN AGING AND OBESITY

There is a relationship between the effects of aging and obesity on the body. Not only can the effects of aging be similar to obesity, aging can even cause obesity development due to decreased metabolic function, decreased muscle mass, and decreased physical activity at an older age.[55] Obesity may even be a symptom of a premature form of metabolic dysfunction, which occurs with age.[56] Some pathologies that aging shares with obesity are steatosis, and increased levels of inflammation and oxidative stress.[55, 57] Aging and obesity can also have similar effects on DNA methylation, especially in blood leukocyte DNA, causing immune system dysfunction.[57] This means that aging can possibly lead to NE-related tissue damage, similar to obesity.

EFFECTS OF AGING ON THE AORTA

The aorta is an elastic artery, and is the largest artery in the body; whose main purpose is to transport oxygenated blood to systemic circulation. The aorta is composed of 3 layers, the tunica adventitia, the tunica media, and the tunica intima. The outermost layer is the tunica adventitia, and is comprised of collagen and elastin fibers, fibroblasts and mast cells.[58] The middle layer is the tunica media, which is the thickest layer. The tunica media is comprised of a large amount of elastin fibers, smooth muscle cells, and collagen.[58] The elastin of the tunica media are also arranged into lamellae, which are concentric layers of elastin.[58] The innermost layer is the tunica intima, which is a single layer of endothelial cells.[58]

When the aorta ages, circumference and diameter are increased.[58] As the aorta ages, elastin fiber fragmentation and lamellae degradation in the tunica media start to occur due to repeated extensions/contractions and oxidative stress.[59] There is an additional increase in collagen as the aorta ages and elastin fragments, with a resulting increase in aorta stiffness.[59] Elastin also undergoes calcification with age due to possessing nucleation sites for apatite minerals, which can also increase aortic stiffness.[59] As aging occurs within the aorta, the balance between proteases and their inhibitors is altered. The release of matrix-metalloproteinases (MMP), inflammatory cell activation, and oxidative stress increases with age, and this in turn induces the release of proteases; like NE.[59] The elastases then act to further break down elastin fibers in the aorta. NE can thus be stimulated by the effects of aging on the aorta, reducing its ability to extend and contract, resulting in aortic tissue damage and fibrosis of the aorta as collagen buildup

occurs. The availability of NE in the aorta is also increased by neutrophil infiltration; which can be caused by ROS or angiotensin II.[59] Increases in ROS and angiotensin II levels occur with age and obesity.[60, 61] Increases in ROS can cause the oxidation of low density lipoprotein (LDL), which can then activate platelets.[62] The activated platelets can then bind neutrophils, forming an aggregate.[62] Activated platelets can then release CCL5 which can bind neutrophils to aortic endothelium, which can result in neutrophil extravasation to the aorta.[62] Angiotensin can induce IL-8 secretion, which can cause neutrophil infiltration, and thus may be another way of neutrophil recruitment to the aorta.[63]

NE has been previously described to be upregulated in obesity, and has its inhibitor A1AT inactivated due to the large availability of ROS in obesity. As NE can also be upregulated in aging, therefore, aged aorta can be another model to study tissue damage caused by NE. Especially as obesity and aging can produce similar pathologies like steatosis, inflammation, oxidative stress, and immune system dysfunction.

METHODS

MICE and FEED CONDITIONS

For the HFHF experiment, same age and sex Wild Type (WT) and Neutrophil Elastase Knockout (NEKO) mice were fed with either normal chow diet (NCD), or high fat diet (HFD, 45 kcal % of fat), or high fat high fructose (HFHFD) for 4 months in Boston University School of Medicine (BUMS) animal facilities where air and light are controlled according to the animal facility regulations.

For the Aging experiment, same sex mice were fed a NCD diet, and WT mice were aged for 2 years, WT-Old. NEKO mice were also aged for 2 years, NEKO-Old. A third group of mice were aged for only 3 months, WT-Young (3 months). Another group of mice, fed a NCD diet aged to 4 months old were also used, WT-Young (4 months), donated by the Seta lab.

TISSUE COLLECTION

At the end of feeding period, mice were sacrificed and mice tissues, such as liver and aorta were extracted and embedded for tissue sectioning.

EMBEDDING

Liver samples were collected in 4% formaldehyde for 24 hours. Tissues were washed with 70% EtOH for 1-2 days. Afterwards, liver samples were placed into cassettes and washed on a shaker in 70% EtOH 2x for 30 minutes each, then 90% EtOH 2x for 30 minutes each, then 95% EtOH 2x for 30 minutes each, then 100% EtOH 2x for

30 minutes each. Next 2x xylene washes were performed for 15 minutes each. Afterwards the samples were washed in liquid paraffin in 60°C for 20 minutes. Then the liver samples were embedded in paraffin using a HistoCore Arcadia H and HistoCore Arcadia C machine and stored in a -20°C fridge.

Aorta samples were extracted from mice and embedded in OCT media, and kept frozen in -80°C fridge.

TISSUE SECTIONING

Liver samples were cut to 14 micrometers on a microtome for use in staining and aorta samples were cut on a cryostat machine to 17 micrometers. Cut tissues were collected on glass slides.

H&E STAINING

Deparaffinization occurred at 60°C for 30 min followed with 2x washes in xylene for 6 minutes each. Rehydration occurred in 2x 6 minute 100% EtOH washes, 2x 6 minute 95% EtOH washes, 2x 6 minute 70% EtOH washes, and 2x 6 minute 50% EtOH washes; followed with a rinse in deionized H_2O for 10 minutes.

Slides were stained with Haematoxylin from Sigma Aldrich for 5 minutes. Slides were then washed in DI H_2O briefly to get rid of excess Haematoxylin. Slides were then dipped in a Differentiation Solution for 1 second. Afterwards the slides were washed in running DI H2O for 10 minutes. Then the slides were washed in 50% EtOH for 5 minutes. Eosin stain from Sigma Aldrich was then done for 3-5 minutes. Afterwards, a

70% EtOH wash was done for 3-5 minutes, followed by a 90% EtOH wash for 3-5 minutes, then a 100% EtOH wash for 3-5 minutes. Then 2x xylene washes were performed for 3 minutes each to wash any excess alcohol.

The slides were then mounted with Vectamount Media.

PICRO-SIRIUS RED STAINING

Deparaffinization occurred at 60°C for 30 min followed with 2x washes in xylene for 6 minutes each. Rehydration occurred in 2x 6 minute 100% EtOH washes, 2x 6 minute 95% EtOH washes, 2x 6 minute 70% EtOH washes, and 2x 6 minute 50% EtOH washes; followed with a rinse in deionized H_2O for 10 minutes.

Liver samples were stained for 1 hour with a 2x Diluted Picro-Sirius Red solution from Poly Scientific R&D Corp. Picro Sirius Red was diluted with ultrapure deionized water and centrifuged at 500g for 5 min before being filtered through a 25mm syringe filter.

5x washes for 10 minutes each in 0.5% Glacial Acetic Acid Water (Dilution of 1 ml of Glacial acetic Acid to 200 ml of ultrapure deionized water).

2-3x washes for 3 minutes each in 100% ETOH. 2-3x washed for 3 minutes each in xylene.

The slides were then mounted in Vectamount media.

IMMUNOHISTOCHEMISTRY

Deparaffinization occurred at 60°C for 30 min followed with 2x washes in xylene for 6 minutes each. Rehydration occurred in 2x 6 minute 100% EtOH washes, 2x 6 minute 95% EtOH washes, 2x 6 minute 70% EtOH washes, and 2x 6 minute 50% EtOH washes; followed with a rinse in deionized H_2O for 10 minutes.

3% H_2O_2 made from a 30% H_2O_2 stock was used to deactivate any peroxidases within the tissue followed by 2x 10 minute rinses in deionized water. Samples were boiled in a hot water bath at 100°C for 25 minutes in a Citrate based antigen unmasking solution from Vector Laboratories to reveal antigen binding sites for antibodies.

3x 5 minute washes in PBS were used to remove any excess unmasking solution. Tissues were then defined by oil with an oil pen to decrease volumes needed for blocking and antibody incubation. A blocking solution consisting of 500 ul of a 1:20 diluted normal goat serum, 10 ul fetal bovine serum, and 1x PBS-Triton (500 ml of 1x PBS to 1.5 ml of Triton) was used to block the tissue samples for 1 hour at room temperature in a humid container.

Primary Antibody was diluted in blocking solution and dropped on tissue samples for overnight binding in the 4°C fridge in a humid container.

Second day samples were taken out of 4°C and left at room temperature for 30 minutes in a humid container. Afterwards, 3x washed for 10 minutes each in 1x PBS-Triton were performed to wash excess primary antibody. Secondary antibody was diluted in blocking solution and left on tissues for between 1 and a half to 2 hours at room temperature in a humid container.

4x washes for 15 minutes each in 1x PBS-Triton were performed to wash away excess secondary antibody. Mounting in Vectamount media from Vector Labs, or DAPI Prolong Gold Antifade from Invitrogen for fluorescent staining was used.

For Co-staining with two different primary antibodies for fluorescence, the above method is followed for the first set of primary and secondary antibody, and then a fix in 4% paraformaldehyde diluted in 1x PBS buffer is performed for 15 minutes. 3x 10 minute washes in PBS-T are then performed to wash off paraformaldehyde. Then the second set of primary antibody is stained overnight. The next day the procedure is followed as normal for washing excess primary antibody and staining secondary antibody. Then mounting is done in DAPI Prolong Gold Antifade media.

For a DAB stain, the above IHC protocol was used, but the secondary antibody is a biotinylated antibody. After 4x 15 minute 1x PBS-Triton washes, streptavidin-HRP is applied to the tissue sample for 30 minutes at room temperature. Then 2x 5 minute 1x PBS-Triton washes were performed and a DAB solution was made with Vector Labs DAB kit. The DAB solution was dropped on the tissue for 10 minutes.

Then 2x 5 minute washes in DI H_20 washes were performed to wash off any excess DAB substrate. 2x Dips in 100% EtOH were done to remove water. 2x dips in 2 changes of Xylene were done to remove alcohol.

Mounting was performed in Vectamount media.

For neutrophil stain, an Anti-Neutrophil Marker antibody from Abcam(ab2557) was used at 1:500 dilution. An Alexa Fluor 594 Goat Anti Rat Red Fluorescent conjugate secondary antibody from Invitrogen(A21213) was used at a 1:800 dilution as a marker.

For F4/80 Stain, an F4/80 antibody from BioRad (# MC497R) was used at a 1:500 dilution. An Alexa Fluor 594 Goat Anti Rat Red Fluorescent secondary antibody from Invitrogen(A21213) was used at a 1:800 dilution as a marker.

For the Anti-Neutrophil Marker with CD4 co-stain, Anti-Neutrophil Marker antibody from Abcam(ab2557) was used at a 1:400 dilution. An Alexa Fluor 594 Goat Anti Rat Red Fluorescent conjugate secondary antibody from Invitrogen(A21213) was used at a 1:800 dilution as a marker. CD4 antibody with an Alexa Fluor 488 conjugate green fluorescent protein from Santa Cruz (sc-19641) was used at a 1:100 dilution.

For the Col1A with DAB stain, the Col1A antibody was from Santa Cruz(sc-59772) and was used at a 1:1000 dilution. A goat anti-mouse biotinylated secondary from Southern Biotech(1031-08) was used as a secondary at a 1:800 dilution. Streptavidin-HRP conjugate from Vector Labs was used directly.

For the elastin stain, elastin antibody from Santa Cruz(sc-58756) was used at a 1:100 dilution. An Alexa Fluor 594 Goat Anti Mouse Red Fluorescent conjugate secondary antibody from Invitrogen was used at a 1:400 dilution.

For the NE stain, NE antibody from Abcam(ab21595) was used as a 1:3000 dilution. A goat anti-rabbit biotinylated secondary from Vector Labs(

TUNEL-DAB STAINING

Adapted protocol from Abcam TUNEL-DAB Kit.

Deparaffinization occurred at 60°C for 30 min followed with 2x washes in xylene for 6 minutes each. Rehydration occurred in 2x 6 minute 100% EtOH washes, 2x 6

minute 95% EtOH washes, 2x 6 minute 70% EtOH washes, and 2x 6 minute 50% EtOH washes; followed with a wash in 1x TBS for 5 minutes.

Tissue was defined by oil with an oil pen to help contain small volumes.

Liver tissue was permeabilized with Proteinase K diluted at 1:100 in distilled DI water for 20 minutes at room temperature. Each sample was covered completely with Proteinase. Then samples were rinsed in 1x TBS for 5 minutes.

Endogenous peroxidases were inactivated with 3% H_2O_2 (30% H_2O_2 was diluted in Methanol) for 5 minutes at room temperature. Next, a wash in 1x TBS was done for 5 minutes.

Liver samples were covered with 100 ul of TdT Equilibration Buffer and incubated at room temperature for 30 minutes.

TdT Labeling Reaction Mixture was made with 1 ul of TdT enzyme to 39 ul of TdT Labeling Reaction Mix. Reaction mixture was added to samples and incubated in a humid container at room temperature for 1.5 hours. Then a wash with 1x TBS was done for 5 minutes.

Stop Buffer was inspected for precipitate and warmed if found until precipitate was gone. Samples were incubated with Stop Buffer for 5 minutes at room temperature to halt the Labeling Reaction. Samples were then washed with 1x TBS for 5 minutes.

The samples were then covered with Blocking Buffer from the kit and incubated at room temperature for 10 minutes.

The Conjugate solution was made by taking the 25x Conjugate and doing a 1:25 dilution in the kit's Blocking Buffer. Samples were incubated for 30 minutes in a humid container at room temperature. Then samples were washed with 1x TBS for 5 minutes.

Working DAB was prepared from the kit DAB Solution 1 and 2. An average of 4 ul of DAB Solution 1 was added to 116 ul of DAB Solution 2 for a 1:30 dilution, though as much was prepared as needed to cover each specimen. The specimens were covered and incubated at room temperature for 15 minutes. Then the samples were rinsed gently with DI water.

The counterstain Methyl Green was placed on the samples to incubate for 1-3 minutes. Then the slides were dipped 2-4 times in 100% EtOH. Afterwards the slides were blotted with a paper towel and slides were dipped 2-4 times in 100% EtOH again. Then slides were dipped 2-4 in xylene and mounted with Vectamount Media.

ALIZARIN RED STAINING

Tissue samples were embedded in OCT, thus no deparaffinization step occurred. Slides were washed in 1x PBS buffer 3x for 5 minutes each. Afterwards, slides were stained in an Alizarin Red solution for 1hr. Alizarin red solution was made from an Alizarin Red powder purchased from Santa Cruz/Chem Cruz. 1 g of Alizarin Red powder was mixed with 100 ml of ultrapure deionized water. The solution was then brought to a pH of 4.2 with a 0.1 M NaOH solution. After 1 hour, slides were washed 3x for 5 minutes in DI H_2O. 2x dips in 100% EtOH were performed to remove excess water. 2x dips in 2

changes of xylene were then performed to remove any 100% EtOH. Slides were then mounted with Vectamount media.

IMAGING

Imaging of tissue was done on a Keyence-9000 microscope with BZ-Viewer software. Images were processed, cropped, and resized through ImageJ.

RESULTS

HFHFD MOUSE MODEL

Mouse models were used to demonstrate the effects of neutrophil elastase on obesity related tissue damage. The mice were split into 4 conditions. The 4 conditions were WT-NCD, WT-HFD, WT-HFHFD and NEKO-HFHFD. Each experiment had between 6-7 mice. NCD mice were fed a normal chow diet pellet. HFD mice were fed a high fat diet pellet. HFHF mice were fed a high fat and high fructose pellet. HFHF(NE KO) mice were mice that had the NE gene knocked out, and were subsequently fed a high fat high fructose pellet. All were fed for a period of 4 months. At the end of their feeding period, mouse livers, adipose tissue, and aortas were extracted for use in staining protocols.

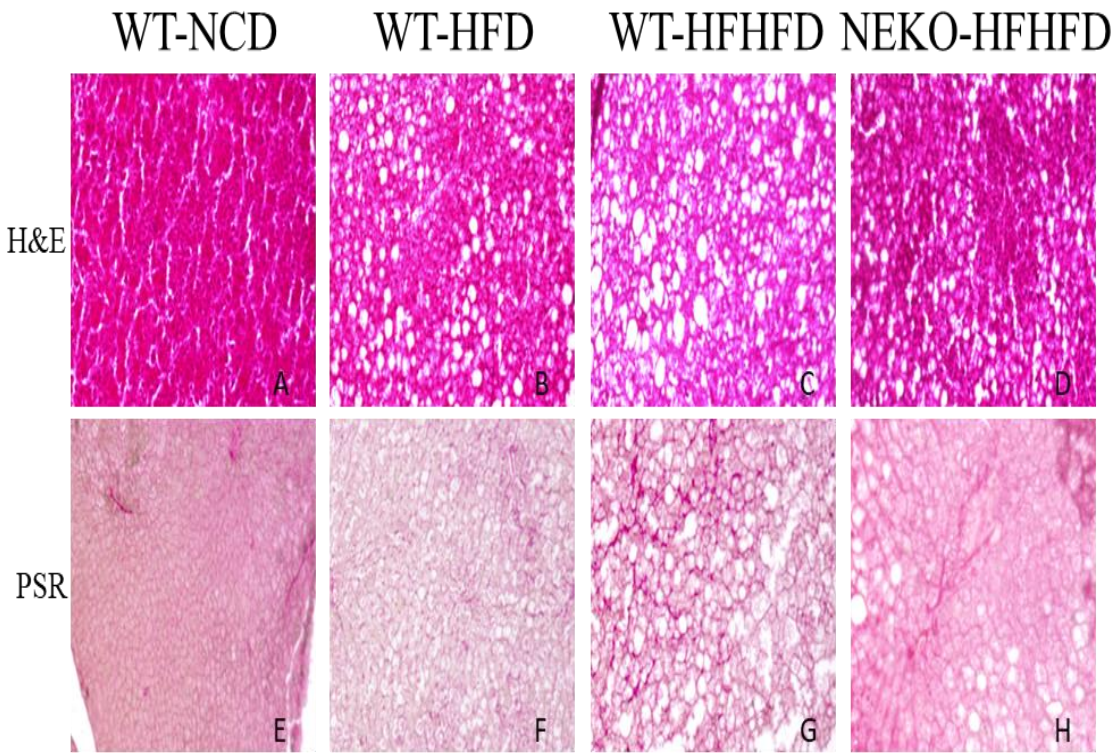
DIET INDUCED DAMAGE IS REDUCED IN NEUTROPHIL ELASTASE KNOCKOUT MICE

To identify the effects of different diets on mouse tissue, Hematoxylin and Eosin stain was performed on liver tissue-sections. WT-NCD mice showed normal liver tissue morphology, with no adipose droplet deposition and no ballooned hepatocytes. WT-HFD mice showed a minimal level of adipose deposition and hepatocyte ballooning, indicating low levels of steatosis. The WT-HFHFD mice had much more severe steatosis compared with the WT-NCD and WT-HFD mice along with hepatocyte ballooning. The adipose droplets in WT-HFHFD were on average larger than adipose droplets found in WT-HFD liver tissue. NEKO-HFHFD mice had less steatosis occur in liver tissue when compared

to WT-HFHFD. Adipose droplet size and hepatocyte ballooning was also smaller when compared to WT-HFHFD. Size of the adipose droplets and hepatocyte ballooning within WT-HFHFD mice was comparable to WT-HFD mice (Figure 2.).

Picro-Sirius Red staining was performed in order to detect the level of fibrosis in the liver tissue of the WT-NCD, WT-HFD, WT-HFHFD, and NEKO-HFHFD mice. WT-NCD mice showed no fibrosis. WT-HFD mice showed extremely low levels of fibrosis. WT-HFHFD mice showed high amounts of liver fibrosis when compared to WT-NCD and WT-HFD mice, especially around areas of lipid droplet deposition. NEKO-HFHFD showed decreased levels of liver fibrosis when compared to WT-HFHFD mice, but higher levels when compared to WT-NCD and WT-HFD mice (Figure 2).

Figure 2. Morphological liver stains of mouse groups



Images taken at 10x magnification. (A-D) H&E(Haematoxyalin and Eosin Stain) to observe morphological changes of liver tissue between the diet groups and effect of NE knockout on mice. (E-H) PSR(Picro-Sirius Red stain) to observe the level of fibrosis between different diet groups and effect of NE knockout on mice.

NEKO-HFHFD mice had decreased morphological damage when compared to WT-HFHFD mice. Their livers were healthier when compared to WT-HFHFD and typically more comparable to WT-HFD with less lipid deposition, smaller lipid droplet size, and less fibrosis compared to WT-HFHFD. It is highly likely that the knockout of

the neutrophil elastase gene grants NEKO-HFHFD mice a protective effect from diet induced tissue damage in mice fed a HFHFD.

HFHFD CAUSES DAMAGE THROUGH NEUTROPHIL ELASTASE RELATED MECHANISMS

To identify the effects of different diets on the level of immune cell infiltration into the liver, immunostaining was performed. Mice fed a HFHF(WT) showed an increase in immune cell infiltration when compared to NCD and HFD(HFD) mice; while HFHF(NE KO) mice showed a decrease in immune cell infiltration compared to HFHF(WT) mice (Figure 3.).

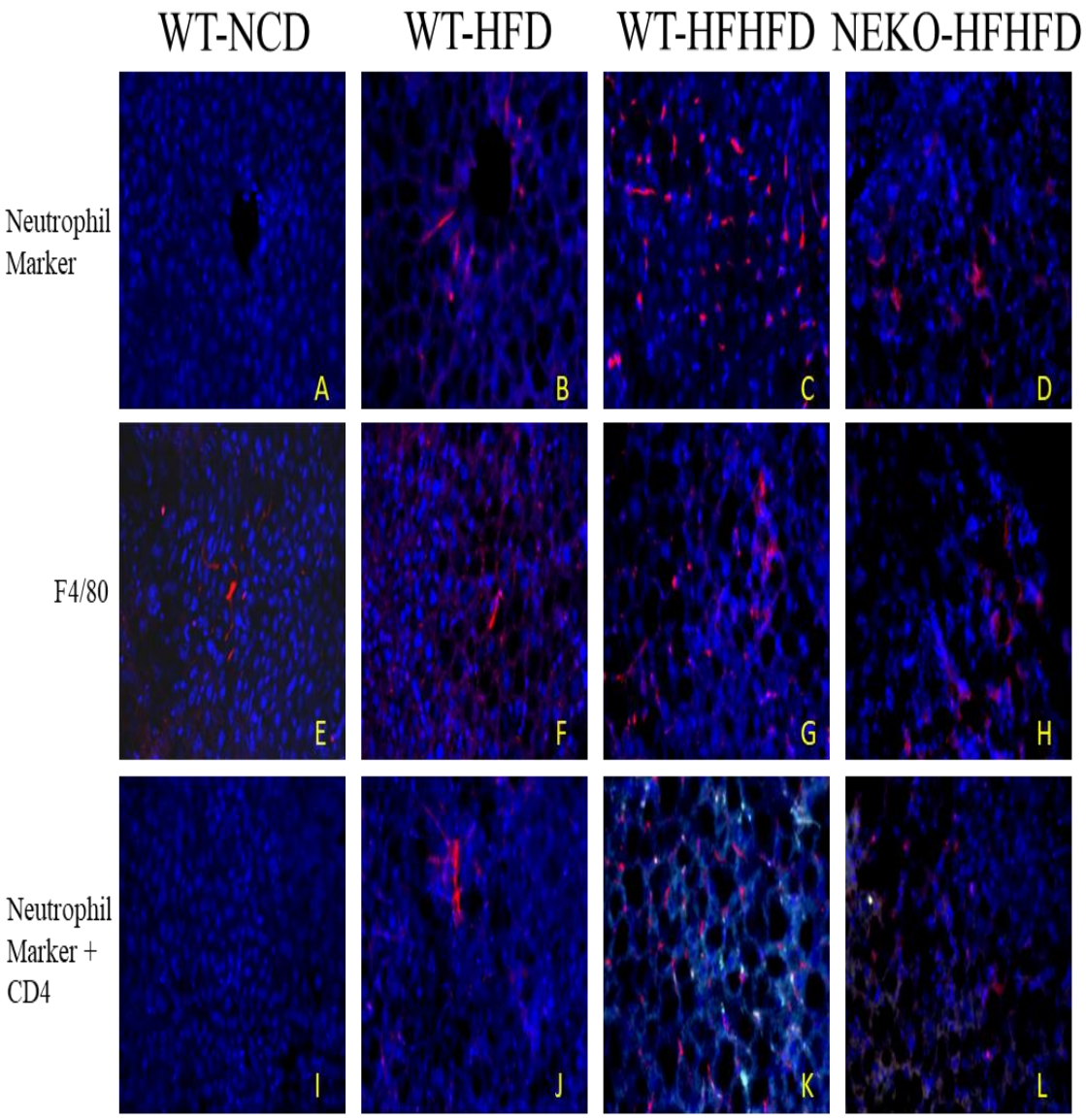
The level of neutrophil infiltration into the liver was examined through immunofluorescent staining with an Anti-Neutrophil Marker antibody with an Alexa Fluor 594 Red Fluorescent Protein attached. NCD mice showed no neutrophil infiltration in the liver, while HFD(WT) mice showed very low levels of neutrophil infiltration into the liver. HFHF(WT) mice however showed significant levels of neutrophil infiltration into the liver. HFHF(NE KO) mice showed reduced levels of neutrophil infiltration into the liver when compared with HFHF(WT) mice, and levels comparable to HFD(WT) mice (Figure 3.).

An F4/80 antibody, a macrophage marker, was also used to stain for macrophages within the liver. An Alexa Fluor 594 Red Fluorescent Protein was attached for detection. WT-NCD and WT-HFD showed comparable levels of macrophages, which are likely resident Kupffer cells. WT-HFHFD showed increased levels of macrophages compared

to WT-NCD and WT-HFD, while NEKO-HFHFD showed reduced levels of macrophages when compared to HFHF(WT) (Figure 3.).

A co-stain of Anti-Neutrophil Marker Antibody with Alexa Fluor 594 Red Fluorescent Protein attached, and CD4 antibody-conjugated to green fluorescent protein, was also performed on mouse liver tissue-sections to examine the amount of interaction occurring between neutrophils and T_h Cells within WT-NCD, WT-HFD, WT-HFHFD, and NEKO-HFHFD groups. WT-NCD mice had no neutrophil and CD4 cell interaction. WT-HFD also had no neutrophil and CD4 interaction. Neutrophil and CD4 interaction was found within the WT-HFHFD group. The NEKO-HFHFD had much reduced levels of neutrophil and CD4 interaction when compared to WT-HFHFD, possibly due to much less infiltration of both neutrophils and CD4+ lymphocytes (Figure 3.).

Figure 3. Immune cell infiltration of mouse liver

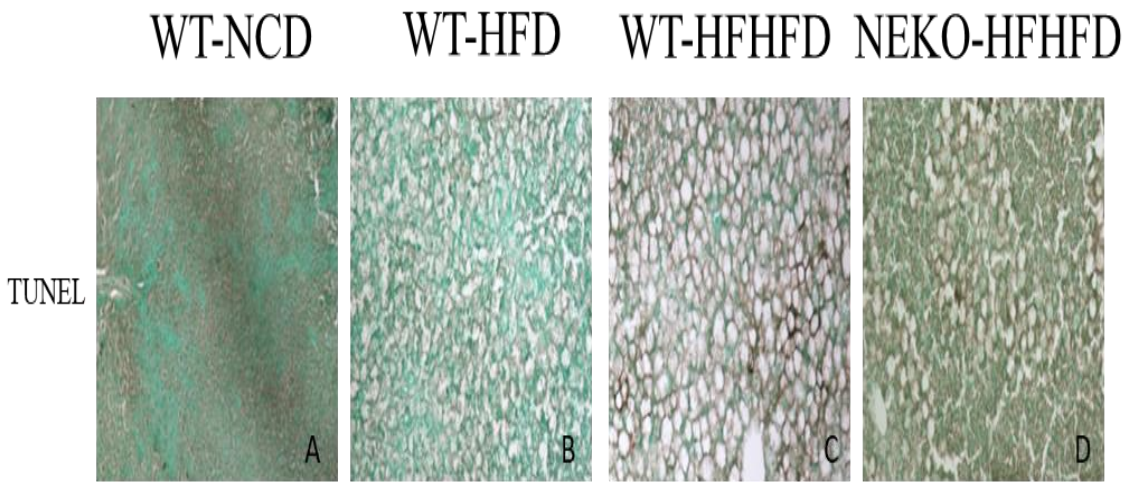


Images taken in 20x magnification. (A-D) Neutrophil infiltration of mouse liver tissue shown with red fluorescent conjugated secondary antibody between different diet groups and effect of NE knockout on mice. (E-H) F4/80, a macrophage marker, used with red fluorescent conjugated secondary antibody to show the amount of macrophages in liver tissue between different diet groups and effect of NE knockout on mice. (H-K) Neutrophil and CD4 co-stain, with neutrophil

marker shown in red fluorescent conjugate secondary antibody and CD4 shown in green fluorescent conjugate. All used a DAPI mounting media to give liver nuclei a blue fluorescence.

To identify the effects of different diets on the level of apoptosis in the liver cells, a TUNEL-DAB assay was performed, with dark brown spots indicating apoptosis. WT-NCD mouse liver showed no apoptosis staining. WT-HFD mouse liver showed no significant levels of apoptosis. WT-HFHFD mouse liver showed increased levels of apoptosis when compared to WT-NCD and WT-HFD mouse livers, especially in areas of lipid deposition. The NEKO-HFHFD mouse liver showed decreased apoptosis when compared to the WT-HFHFD group, but still an increased level when compared to WT-NCD and WT-HFD (Figure 4.).

Figure 4. Apoptosis of mouse liver



Images taken in 10x magnification. (A-D) Tunel-DAB assay performed on liver tissue to ascertain the level of apoptosis between different diets and effect of NE knockout.

NEKO-HFHFD mice have reduced levels of immune cell infiltration and apoptosis when compared to WT-HFHFD mice. The level of neutrophils, T_h Cells, and macrophages was lower compared to WT-HFHFD mice, and more comparable with WT-HFD mice. Apoptosis also decreased in NEKO-HFHFD mice, indicating healthier liver when compared to WT-HFHFD mice. This once more indicates that knockout of NE provides a protective effect for mice who are fed HFHFD.

Based on the results of tissue staining and published literature information described above, the mechanism for NE induced tissue damage is proposed as following. HFHF diet results in the development of lipid droplets within the liver due to increased Methylglyoxal levels, which block AMP and AMPK interaction, allowing acetyl CoA carboxylase to generate an increased number of fatty acids. Increases in fructose and fat

content in the liver also lead to the generation of ROS, ceramides, IL-6, TNFα, and a depletion of ATP; which cause liver inflammation and tissue damage, causing the beginning stages of NASH. The increase in lipid droplets stimulate neutrophil infiltration due to an increase in chemokines production. HFHFD increasing levels of CD11b in neutrophils will also stimulate neutrophil infiltration into the liver. In addition, the results from HFHF fed WT mice show increased differentiation of Th-17 cells, a CD4+ T_h Cell subset that also works to increase neutrophil infiltration into the liver through IL-17 release. The increased level of neutrophil infiltration and stimulation in the liver would in turn increase the availability of NE. Due to the increased levels of ROS from neutrophils due to HFHF diet, A1AT activity is inhibited, and NE is able to cause damage to the surrounding tissue, breaking down extracellular matrix and cadherins. MPO release is also stimulated, which can work to further stimulate neutrophils, resulting in a continuous cycle of increased neutrophil activity and NE availability. Neutrophil elastase will then increase the expression of inflammatory cytokines like TNFα, which will in turn work to increase neutrophil recruitment into the liver. NE also causes increased lipid accumulation in the liver, and the increased synthesis of ceramides through increasing the expression of serine palmitoyltransferase. The tissue damage caused by the long-term activation of neutrophils and NE results in apoptosis of hepatocytes and the activation of hepatic stellate cells. NE also activates pro-fibrotic cytokines such as IL-1β and IL-33, which will aid in stimulating hepatic stellate cells. Activated hepatic stellate cells will then accumulate collagen fibers, leading to fibrosis and eventual cirrhosis of the liver in NASH.

AGING MOUSE MODEL

To examine the effect of aging on neutrophil elastase related tissue damage and relate to obesity damage, 5 mice were split into 3 groups. One group of WT mice (WT-Old) were aged for 2 years and fed a normal chow diet. Another group of mice had the gene NE deletion (NEKO-Old) and were also fed a normal chow diet, and aged for 2 years. Another group of WT mice was aged to 3 months old and fed a normal chow diet, WT-Young (3M). The mice were sacrificed, and their aortas extracted for use in histological protocols. A different group of WT mice aged to 4 months old, WT-Young (4M) were also used in histological protocols, and were donated by the Seta Lab.

AGING CAUSES DAMAGE SIMILAR TO OBESITY THROUGH NEUTROPHIL ELASTASE RELATED MECHANISMS

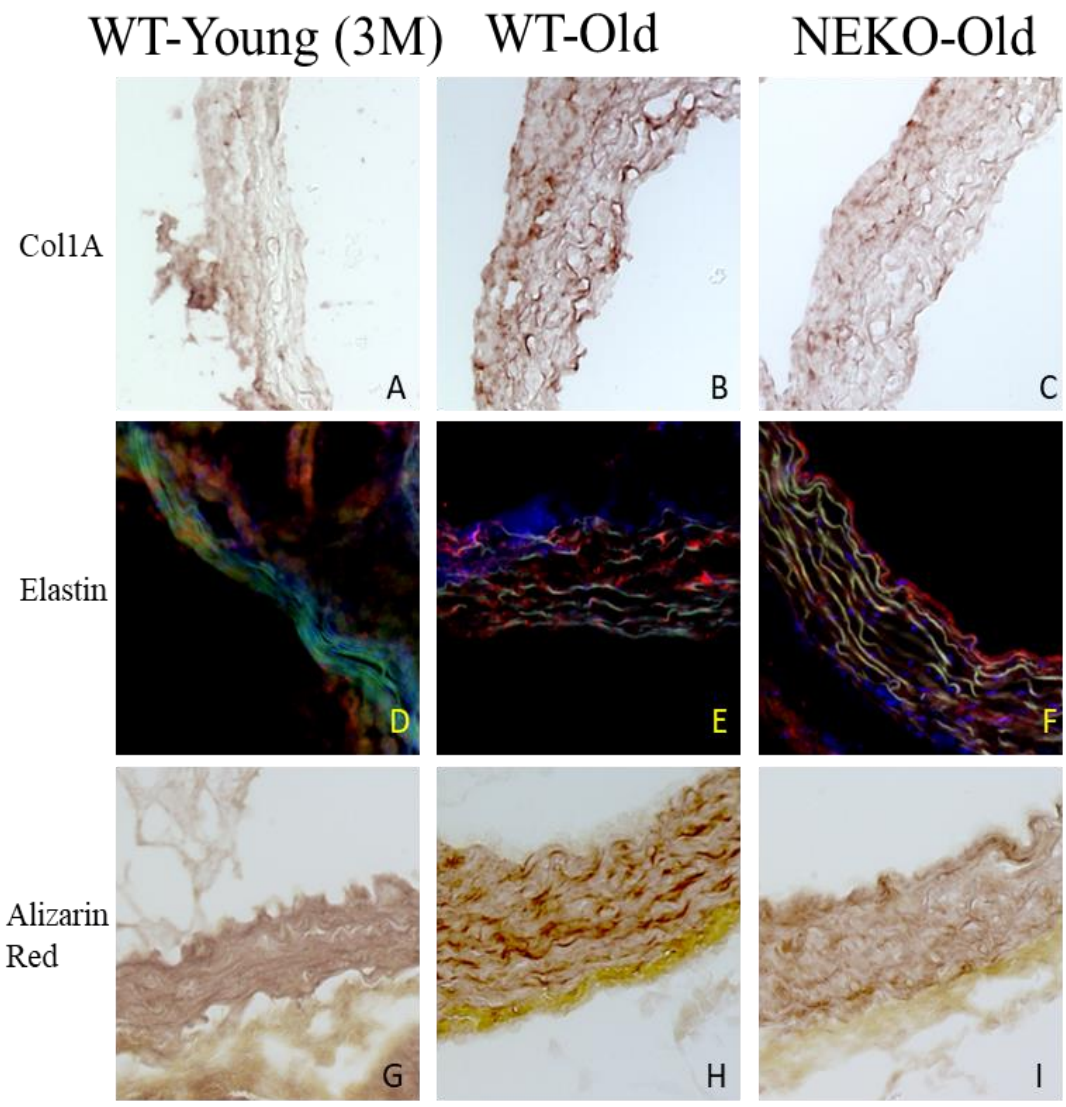
To identify the effect of aging on mouse aorta health and compare to obesity, immunostaining was performed. A DAB stain was used with Col1A antibody to detect the level of collagen deposition in the aorta of WT-Old, NEKO-Old, and WT-Young (3M) mice. The WT-Young (3M) mouse aorta had low collagen content within the aorta. Much higher collagen accumulation was observed within WT-Old mouse aorta, indicating fibrosis within the WT-Old mouse aorta. The NEKO-Old mouse had a decreased amount of collagen when compared to the WT-Old mouse, indicating a protective effect of NE deletion on aging related fibrosis knockout (Figure 5.).

An elastin antibody with red fluorescent protein was used to stain for elastin content within the aorta; while green fluorescence was used as an additional method to

observe elastin due to green autofluorescence. The red fluorescence showed a high amount of background for the elastin stain, and it was difficult to isolate the elastin fibers in red fluorescence. However, elastin fibers were easily viewed under green fluorescence, allowing for some analysis of elastin integrity. WT-Young (3M) mouse aorta had intact, long strands of elastin with lamellae. WT-Old mouse aorta had short, discontinuous strands of elastin fibers, indicative of elastin breakdown with aging. NEKO-Old mouse aorta showed intact elastin fibers similar to WT-Young (3M) mice, with only occasional fragmentation, once more showing a possible protective effect of NE deletion (Figure 5.).

An Alizarin Red stain was used to detect the level of calcification in mouse aorta. WT-Young (3M) mouse aorta showed no calcium deposition. WT-Old mouse aorta showed the highest amount of calcium deposition, especially in locations similar to elastin fibers. NEKO-Old mouse aorta showed a dramatically decreased level of calcium deposition within when compared to WT-Old mouse aorta (Figure 5.).

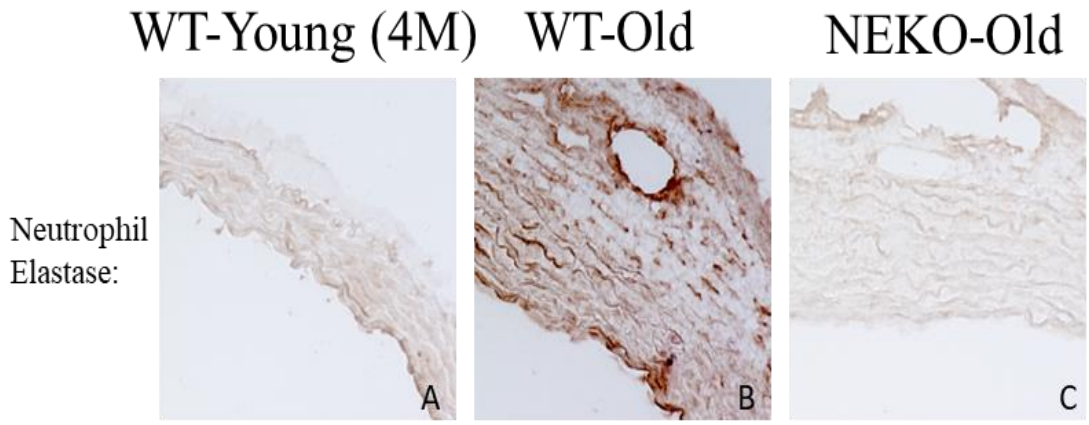
Figure 5. Effect of aging on mouse aorta



Images taken in 20x magnification. (A-C) Collagen 1 with DAB staining to examine the level of fibrosis in young vs aged mouse aorta vs knockout mice. (D-F) Elastin staining with red fluorescent conjugate secondary antibody merged with green autofluorescence of elastin to examine the degree of elastin fragmentation in young vs aged mouse aorta vs knockout mice. (G-I) Alizarin Red staining on aged mouse aorta to examine the degree of calcification of young vs aged mouse aorta vs knockout mice.

A DAB stain was used with NE antibody in order to detect the level of NE within the aorta of WT-Old, NEKO-Old, and WT-Young (4M) mice. WT-Young (4M) mice showed no NE within the aorta. WT-Old mice showed a high level of NE when compared to WT-Young (4M) mice. NEKO-Old mice showed no sign of NE within the aorta, comparable to WT-Young (4M) mice and indicating functional deletion of NE (Figure 6.). The presence of NE within WT-Old mice and lack of NE within WT-Young (4M) and NEKO-Old indicates NE related tissue damage when compared to prior stains.

Figure 6. Level of NE within aortic tissue



Images taken in 20x magnification. (A-C) NE staining with DAB performed to ascertain the level of NE between young vs aged vs knockout mice.

Based on our results and other publications, the following is a proposed mechanism for NE related tissue damage in aged mouse aorta that relates to obesity. Similar to obesity, aging also increases the level of ROS, Angiotensin II, and IL-8, causing neutrophil infiltration into the aorta due to platelet complexes and Angiotensin II

induced IL-8 release. The infiltration of neutrophils into the aorta causes an increased availability of NE. NE has increased activity due to the inactivation of A1AT inhibitor because of high ROS. NE will degrade elastin fibers; fragmenting them, and likely exposing more nucleation sites for apatite minerals, thus increasing calcification of the tunica media in aged mice. The damage caused by NE stimulates fibroblasts of the tunica adventitia, which could migrate to the tunica media and begin to deposit collagen fibers, causing fibrosis. The calcification and fibrosis of the aorta increase aortic stiffness. The lack of ability to extend and contract due to decreased elastin content, fibrosis, and calcification can increase the risk of further damage to the aorta during aging.

DISCUSSION

SUMMARY

Obesity is a major worldwide health concern. As the issue grows, it becomes of greater importance to understand the mechanisms behind which obesity causes tissue damage. Neutrophils and neutrophil elastase are one component through which obesity is capable of causing tissue damage. Through tissue staining of the liver and aorta, the goal was to discover possible mechanisms through which neutrophils and neutrophil elastase cause damage to tissue in obesity and aging. In our experiment we had 4 different mouse groups based on diet and gene expression. WT-NCD were wild-type mice fed a normal chow diet, WT-HFD were wild-type mice fed a high fat diet, WT-HFHFD were mice fed a high fat high fructose diet, and NEKO-HFHFD were mice that had the gene NE deleted and were fed a high fat high fructose diet. These mice had their livers extracted, embedded in paraffin, and had histological protocols performed.

Mouse liver tissue from these experiments had H&E and Picro-Sirius Red staining performed to examine morphological changes to the liver tissue. Fluorescence staining was used to observe immune cell infiltration of the liver. The cells examined for infiltration were macrophages, neutrophils, and T_h cells. TUNEL-DAB staining was used to examine the level of apoptosis that occurred in the liver of the different mouse diet groups.

Another experiment to examine the effect of aging on tissue damage had mice three groups of mice. WT-Young (3M) mice were wild type mice that were 3 months old and fed a normal chow diet, while WT-Young (4M) were similar, but aged to 4 months.

WT-Old mice were mice that were 2 years old and fed a normal chow diet. NEKO-Old mice were mice that were 2 years old; had the gene NE deleted, and were fed a normal chow diet. These mice had their aorta's extracted, embedded and frozen in OCT, and had histological protocols performed.

Mouse aortic tissue from this experiment had immunostaining with DAB performed with a Col1A antibody to detect the level of collagen between groups. A fluorescent stain was performed to stain for elastin in order to observe the difference in elastin integrity between the groups. Alizarin Red Staining was used to observe the level of calcification that occurred in aorta between the mouse groups.

Mouse liver tissue from the HFHFD experiment showed that a HFHFD diet caused increased lipid deposition, fibrosis, neutrophil, macrophage, and T_h Cell infiltration within the liver when compared to a HFD. HFHFD with NEKO mice showed that the deletion of NE results in a protective effect, as these mice had less adipose buildup in their liver, less fibrosis, and a decreased level of immune cell infiltration. HFHFD increases lipid deposition in the liver and stimulates immune cell infiltration into the liver. Neutrophils and NE are upregulated in HFHFD and elastase activity is increased due to the inhibition of A1AT, a NE inhibitor. The increased activity of NE increases tissue damage and will promote fibrosis of the liver in NASH.

From the aging experiment, we showed that aged mice, which were used as a comparison to obese mice, had more severe aortic damage as compared to similarly aged mice that had the NE deleted. WT-Old mice had more collagen deposition in the aorta, a greater degree of fragmentation of their elastin fibers, and more calcium buildup as

compared to NEKO-Old mice and WT-Young mice. WT-Old mice were also the only group with the presence of NE. This indicates that knocking out NE can offer a protective effect from the pathologies of aging. With the pathology of aging being similar to obesity, knockout of NE would likely offer a similar degree of protection to the aorta from damage.

The knockout of NE shows a reduction in the negative effects of obesity from HFHFD. Liver and aortic tissue is healthier. The mechanism for NE related damage can be triggered by a HFHFD, which is common in the United States, and is becoming more common worldwide, leading to increased obesity. HFHFD stimulates neutrophil infiltration into the liver and enhances neutrophil activity, increases ROS which deactivates A1AT, decreasing the inhibition of NE. MPO release is also stimulated, which also enhances neutrophil activity. Macrophages are also activated, and along with T_h Cells further stimulate neutrophil activity and infiltration. The increased availability of NE results in more tissue damage, which leads to eventual fibrosis of the tissue.

REMAINING QUESTIONS

While the mechanism for stimulating neutrophil and neutrophil elastase induced tissue damage has been explored, a deeper investigation can be done to understand more about how neutrophils and neutrophil elastase can cause damage in obesity. Are there any specific cell markers that neutrophils and neutrophil elastase target which are upregulated in obesity? Does neutrophil elastase stimulate other immune cell activity? Does neutrophil elastase upregulate additional genes besides serine palmitoyltransferase? Are

there products left behind by neutrophil elastase that may cause additional tissue damage in obesity? Are there additional cytokines upregulated in obesity that can enhance neutrophil elastase stability and increase its activity? These questions can help to further understand the mechanisms behind neutrophil and neutrophil elastase related tissue damage. This will provide deeper understanding of obesity as a disease, and how it may be better treated.

LIMITATIONS

There was an attempt to stain liver tissue from the HFHFD experiment with NE antibody with a red fluorescent conjugated secondary antibody. However, the antibody failed to stain properly and no proper signal could be differentiated from tissue background. Thus neutrophil marker antibody was used instead, as deletion of NE reduces neutrophil function and has impact on infiltration into the liver. Therefore, it was used as a marker of both neutrophil and NE activity. Time constraints prevented further testing with newer NE antibody. Though in the future a NE stain will need to be performed to gain a more accurate idea of the activity of NE in the liver under WT-HFD and WT-HFHFD conditions.

Another ideal stain to perform on liver tissue from the HFHFD experiment would have been a co-stain using F4/80 and CD11c. This would have allowed for the differentiation between blood borne macrophages and resident Kupffer cells of the liver. This could have shown if neutrophil elastase in WT-HFHFD increases the infiltration of

blood borne immune cells into the liver. However, time constraints prevented this specific co-staining of the liver.

Elastin images also showed a high background and low red fluorescence specificity for elastin for WT-Young (3M) mouse aorta. Mouse aorta for WT-Young (3M) mice was also found to have many mis-folds and other artifacts from cryostat sectioning. Improvements to sectioning technique need to be made for proper tissue sectioning and placement on slides. The primary elastin antibody dilution of 1:100 was too high and may need further dilutions, such as 1:500, and 1:1000 in order to attain better signaling. The secondary red fluorescent antibody dilution of 1:400 was also too high, and further dilutions such as 1:800, or 1:1500 may be required. Increased washes in between primary and secondary antibodies will also be needed to help improve signal specificity, as well as increasing the speed of washes.

Aortic tissue from the HFHFD experiment would have been ideal to use for staining. Aortic tissue from HFHFD experiment could have presented a more accurate idea of neutrophil and NE activity and tissue damage in obesity. Useful comparisons could have also been made between the aging experiment mouse aorta, and HFHFD experiment mouse aorta to further elucidate the similarities between aging and obesity. However, inexperience in cutting cryostat sections and time constraints prevented sectioning of more aorta samples for examination.

FUTURE DIRECTIONS

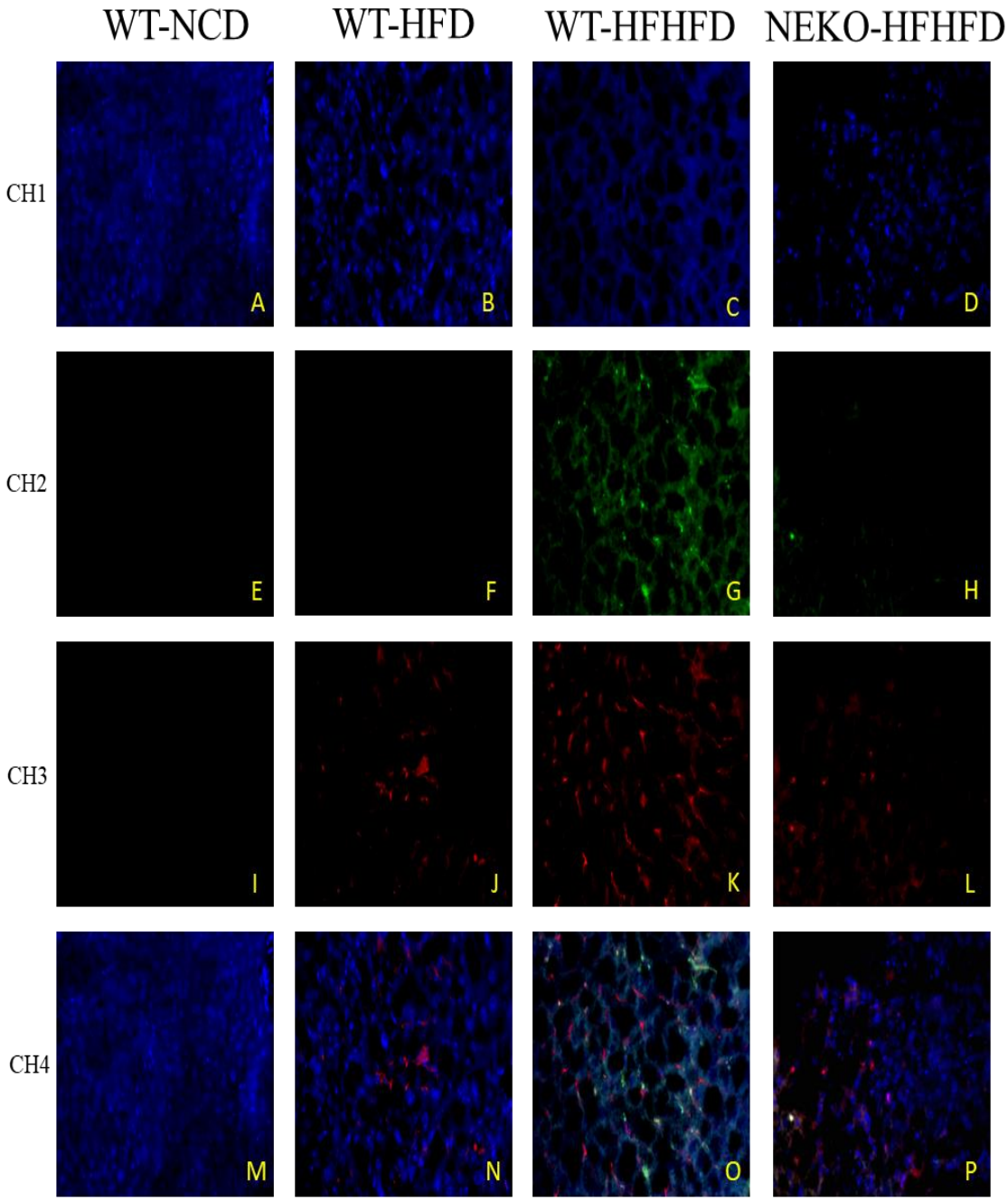
While it may be possible to inhibit neutrophil elastase as a treatment for obese patients, long term this may be inadvisable, as neutrophil elastase plays an important role in antimicrobial defense. It may be of interest to investigate the early triggers and stimulators of neutrophils and NE; such as IL-8 and ROS, and develop treatments that reduce the levels of these promoters of neutrophil and neutrophil elastase activity.

Future studies could therefore be aimed at discovering and inhibiting the stimulators of neutrophils and neutrophil elastase. Future studies should also aim towards understanding further interactions between neutrophils, NE, other immune cells, and possibly inhibiting those interactions. There could also be investigation into what other genes NE may upregulate. Studies developed towards inhibiting the generation of toxic byproducts of high fructose consumption, such as ceramides may also prove useful in reducing the damage neutrophil elastase may cause.

Neutrophils and the enzyme NE are an important part of our immune system. When properly functioning, they offer a vital defense against dangerous microbial infections. However, in obesity, neutrophil elastase causes tissue damage, harming the ability of the body to function properly. While some understanding into the mechanisms of neutrophil elastase related damage has been gained, it is important that continued studies are performed in order to deepen our understanding of how and why neutrophil elastase causes obesity related damage.

APPENDIX

Figure 7. Neutrophil and CD4 infiltration of liver



Neutrophil and CD4+ costain of mouse liver tissue-section from HFHFD experiment. (A-D) CH1 shows blue fluorescence for all diet groups, illuminating liver nuclei. (E-H) CH2 shows CD4 staining with green fluorescent conjugate for all diet groups. (I-L) shows Neutrophil Marker staining with red fluorescent conjugate secondary antibody for all diet groups. (M-P) CH4 shows merged channels for all diet groups.

LIST OF JOURNAL ABBREVIATIONS

Adv Nutr	Advances in Nutrition
Anat Cell Biol	Anatomy and Cell Biology
Cell Metab	Cell Metabolism
Cell Mol Gastro Hepatol	Cellular and Molecular Gastroenterology and Hepatology
Cell Mol Immunol	Cell & Molecular Immunology
Clin Exp Immunol	Clinical and Experimental Immunology
Clin Liver Dis	Clinics In Liver Disease
Curr Pharm Des	Current Pharmaceutical Design
Front Immunol	Frontiers in Immunology
Front Physiol	Frontiers in Physiology
Gastro Hepatol	Clinical Gastroenterology and Hepatology
Int J Environ Res Pub Heal	International Journal of Environmental Research and Public Health
J Biol Chem	Journal of Biological Chemistry
Mol Cell Biol	Molecular and Cellular Biology
Mol Med	Molecular Medicine
Nat Med	Nature Medicine
Pharmacol Rev	Pharmacological Reviews
Physiol Rep	Physiological Reports
PLoS One	Public Library of Science

Popul Health Metr Population Health Metrics

Proc Natl Acad Sci USA Proceedings of the National Academy of Sciences of the United States of America

Oxid Med Cell Longev Oxidative Medicine and Cellular Longevity

World J Hepatol World Journal of Hepatology

World J Gastro World Journal of Gastroenterology

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