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# Effects of aging and diet on neprilysin activity

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**EFFECTS OF AGING AND DIET ON NEPRILYSIN ACTIVITY**

by

**ETHAN KALLICK**

B.A., Davidson College, 2014

Submitted in partial fulfillment of the  
requirements for the degree of

Master of Science

2019

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## **DEDICATION**

I would like to dedicate this work to Dr. Colucci's lab at Boston Medical Center

## **ACKNOWLEDGMENTS**

I would like to sincerely thank Dr. Wilson Colucci, M.D., Dr. Deborah Siwik, Ph.D., Dominique Croteau, and all of the other members of Dr. Colucci's Myocardial Biology Laboratory at Boston Medical Center. Thank you so much for all of your help and guidance, and for allowing me to be part of this research.

## **EFFECTS OF AGING AND DIET ON NEPRILYSIN ACTIVITY**

**ETHAN KALLICK**

### **ABSTRACT**

Neprilysin (NEP) is an endopeptidase with several vasoactive substrates, including natriuretic peptides. A NEP inhibitor combined with an angiotensin receptor blocker (LCZ696) improves outcomes in patients with heart failure with reduced ejection fraction (HFrEF); however, it has not been tested in patients with HF with preserved ejection fraction (HFpEF). Two common forms of HFpEF are caused by aging and metabolic syndrome (MetS)/type 2 diabetes. Therefore, the goal of this study was to determine whether NEP activity is increased in mice with diastolic dysfunction due to aging or MetS.

C57BL/6J mice were fed normal chow diet (NCD) for the first two months of their life, before being switched to either control diet (CD) or a high fat, high sucrose (HFHS) diet for 4 or 16 months, which allowed for determination of the effects of both age and diet on NEP activity. NEP activity was measured in plasma based on the generation of a fluorescent product, methoxy-2-naphthylamine (MNA). Samples were incubated +/- NEP inhibitor thiorphan to distinguish NEP activity from activity of other non-specific endopeptidases. The difference in fluorescence of MNA was then measured, and samples incubated with thiorphan were subtracted from samples incubated without thiorphan to give plasma NEP activity. Baseline NEP activity values (BL,  $5.0 \pm 2.9$ ) were derived from 2 month old mice fed Normal Chow Diet (NCD). Several mice were not switched to CD or HFHS, but were instead kept on NCD for an additional 4 months to act

as an age-matched control against the CD cohort, for determination as to whether CD had a differential effect on NEP activity relative to NCD. The mice switched to CD had increased NEP activity at 6 months compared to NCD-fed mice ( $14.8 \pm 6.4$ ;  $p < 0.0002$  vs. BL). For this reason, mice were fed CD for all subsequent experiments. NEP activity in CD-fed mice increased from 4 months to 16 months ( $14.8 \pm 6.4$  vs.  $65.3 \pm 16.4$ ;  $p = 0.0028$ ). NEP activity in HFHS-fed mice increased from 4 months to 16 months as well ( $20.0 \pm 7.6$  vs.  $43.8 \pm 20.9$ ;  $p = 0.0031$ ) Comparison of the age-matched effect of HFHS diet relative to CD revealed that there was a trend towards significance at 4 months ( $20.7 \pm 7.6$ ;  $p = 0.06$  vs. CD) but not at 16 months ( $43.8 \pm 20.9$ ;  $p = \text{ns}$  vs. CD).

This study revealed that both CD and HFHS increase NEP activity compared to NCD, perhaps as a result of the lard (saturated fat) as the principal source of fat in those diets. More importantly, it revealed that NEP activity increases with aging, and that aging exerts a greater influence on NEP activity than diet (MetS) does. Finally, it was shown that LCZ696 can effectively lower plasma NEP activity. Mice fed CD and HFHS diet for 16 months that received one week of 100 mg/kg LCZ696 therapy had 78% inhibition ( $p = 0.0286$ ) and 85% inhibition ( $p = 0.0006$ ) of their plasma NEP (sNEP) activity, respectively. This strongly suggests the utility of LCZ696 as a promising treatment option for patients with aging and MetS-related HFpEF.

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## LIST OF ABBREVIATIONS

ACEI.....	ACE Inhibitor
ARB.....	Angiotensin Receptor Blocker
ANP.....	Atrial Natriuretic Peptide
APM.....	Aminopeptidase M
BL.....	Baseline
BMI.....	Body Mass Index
BNP.....	B-type Natriuretic Peptide
CNP.....	C-type Natriuretic Peptide
CSF.....	Cerebrospinal Fluid
cGMP.....	Cyclic Guanosine Monophosphate
CO.....	Cardiac Output
DMF.....	Dimethyl Formamide
DTT.....	Dithiothreitol
ECM.....	Extracellular Matrix
ECV.....	Extracellular Volume
EF.....	Ejection Fraction
FA.....	Fatty Acid
Glu-MNA.....	Glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamine
HF.....	Heart Failure
HFD.....	High Fat Diet
HFHS.....	High Fat High Sucrose Diet

HR.....	Heart Rate
HFrEF .....	Heart Failure with Reduced Ejection Fraction
HFpEF.....	Heart Failure with Preserved Ejection Fraction
LA.....	Left Atrium
LV .....	Left Ventricle
MMPs.....	Matrix Metalloproteinases
MetS.....	Metabolic Syndrome
MI.....	Myocardial Infarction
MNA.....	Methoxy-2-naphthylamine
mRNA.....	Messenger RNA
NCD.....	Normal Chow Diet
NEP .....	Neprilysin
NO.....	Nitric Oxide
NP.....	Natriuretic Peptide
NPR.....	Natriuretic Peptide Receptor
NPR-A.....	Natriuretic Peptide Receptor-A
NPR-B.....	Natriuretic Peptide Receptor-B
NPR-C.....	Natriuretic Peptide Receptor-C
PKG.....	Protein Kinase G
RAAS.....	Renin-Angiotensin-Aldosterone System
ROS.....	Reactive Oxygen Species
sGC.....	Soluble Guanylate Cyclase

sNEP.....	Soluble Neprilysin
SNS.....	Sympathetic Nervous System
SV.....	Stroke Volume
T2DM.....	Type 2 Diabetes Mellitus
TIMPs.....	Tissue Inhibitors of Matrix Metalloproteinases
VCAM.....	Vascular Cell Adhesion Molecule
WT.....	Wild Type

## INTRODUCTION

Neprilysin (NEP) is a membrane-bound, zinc-dependent endopeptidase that is expressed ubiquitously including in heart, brain, peripheral nerves, lungs, vasculature, adrenal glands, lungs, epithelia, adipocytes, fibroblasts and neutrophils. Membrane-bound NEP has a large extracellular catalytic domain, a transmembrane region, and a short cytoplasmic N-terminal domain (Erdos & Skidgel, 1989; Maguer-Satta, Besançon, & Bachelard-Cascales, 2011). Importantly, NEP exists in soluble form (sNEP) in plasma, Cerebrospinal Fluid (CSF), and urine (Bayes-Genis, Barallat, & Richards, 2016), and retains its catalytic activity (Spillantini et al. 1990; Yandle et al. 1992). The process whereby NEP goes from its tissue-based, membrane-bound form to become solubilized (sNEP) is ultimately unknown, but it is theorized that NEP undergoes ectodomain shedding, which entails proteolytic cleavage of its extracellular domain (Bayes-Genis, 2015). In addition to the soluble form (sNEP), NEP in plasma exists on the neutrophil plasma membrane where it cleaves the chemotactic peptide fMet-Leu-Phe, and likely has a role in modulating neutrophil chemotaxis (Roques et al. 1993; Connelly et al. 1985). NEP is also referred to as neutral endopeptidase, CD10, enkephalinase, common acute lymphoblastic leukemia antigen, and endopeptidase 24.11 (Bayes-Genis, 2015); however, for the purposes of this thesis, it will be referred to solely as Neprilysin (NEP).

NEP is composed of 749 amino-acid residues (Oefner et al. 2000). NEP is encoded by chromosome 3 (3q21-27) and spans more than 80 kb, with its messenger RNA (mRNA) containing 24 exons (Li et al. 1995; Tiraboschi et al. 1999; D'Adamio et al. 1989). Notably, the peptide sequence His-Glu-Ile-Thr-His is responsible for enzyme

binding and substrate catalysis, and is encoded within exon 19 (D'Adamio et al. 1989).

NEP contains a single zinc atom, coordinated by residues of Histidine and Glutamic acid, and is crucial for catalytic activity. NEP is restricted by its small catalytic site to cleaving small peptides with a molecular weight below 3,000 Da (Pankow et al. 2009).

Proteolytic activity has been characterized as NEP having a special preference for the amino side of hydrophobic amino acids; specifically, Phenylalanine or Leucine at the P1 position (Bayes-Genis, Barallat, & Richards, 2016).

NEP has over 50 substrates, both *in vitro* and *in vivo* (table 1) (Bayes-Genis, Barallat, & Richards, 2016); given the wide-ranging distribution of NEP in various tissues coupled with its myriad substrates, it is clear that NEP plays a role in both health and disease. Within the context of Heart Failure (HF), Atrial Natriuretic Peptide (ANP), B-type Natriuretic Peptide (BNP), C-type Natriuretic Peptide (CNP), Angiotensin 2, Endothelin, Bradykinin, and Adrenomedullin are NEP's salient substrates and will therefore, be the ones focused on. Much of the clinical impact of NEP inhibition is assumed to be due to augmentation of Natriuretic Peptide (NP) activity (Bayes-Genis, Barallat, & Richards, 2016) and as such, special attention will be given to the natriuretic peptides (NPs) given their known cardiovascular effects.

<b>Cardio-renal</b>	<b>ANP, BNP, CNP; Angiotensin 1, 2, 3, 1-9; Endothelin-1, -2, -3; Adrenomedullin, Bradykinin</b>
<b>Nervous System</b>	<b>Amyloid-beta; Enkephalins; alpha-Endorphin, gamma-Endorphin; alpha-Neoendorphin, beta-Neoendorphin; Nociceptin; Corticotropin-releasing factor; Luteinizing hormone-releasing hormone; Oxytocin; Antidiuretic Hormone; Neurotensin; Neuropeptide Y</b>
<b>Gastrointestinal</b>	<b>Gastrin-releasing peptide; Gastric inhibitory peptide; vasoactive intestinal peptide; cholecystokinin</b>
<b>Respiratory</b>	<b>Substance P</b>
<b>Metabolic</b>	<b>Glucagon; Glucagon-like peptide; beta-lipotropin; insulin beta-chain; secretin; somatostatin</b>

**Table 1. Neprilysin substrates.** Neprilysin (NEP) has various substrates in multiple tissues (adapted from Bayes-Genis, Barallat, & Richards, 2016).

## **Natriuretic Peptides**

There are three major natriuretic peptides (NPs): atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). NPs are released in response to pressure and volume overload, and act to protect the cardiovascular system from the deleterious effects of such hemodynamic overload by swiftly inducing diuresis, natriuresis, kaliuresis, vasodilation, and inhibiting the renin-angiotensin-aldosterone system (RAAS). It is well known that tonic activation of RAAS leads to pathologic cardiac remodeling via induction of cardiac myocyte hypertrophy, and interstitial fibrosis (Kim et al. 1995; Konstam et al. 1992); therefore, NPs play a very important counterregulatory role in preventing RAAS-mediated pathology and maintaining homeostasis. Several models elucidate this point via inactivation of NP bioactivity, either via NP-specific knockout or NP receptor knockout model, showing the deleterious cardiac effects of NP insufficiency. An ANP receptor (NPR-A) knockout mouse model showed significantly depressed systolic and diastolic function, interstitial fibrosis, and a shift in cardiac gene expression towards upregulation of pro-hypertrophic markers correlating to profound cardiac hypertrophy that resulted in a 61% increase in heart weight compared to control (Kuhn et al. 2002). Similarly, exogenous BNP administration to HF patients in a number of randomized, controlled clinical trials resulted in a number of improvements in cardiac parameters including decreases in right atrial pressure, pulmonary artery pressure, pulmonary wedge pressure, mean arterial pressure, pulmonary/vascular resistance, and increased cardiac index as well as improvements in HF symptoms such as dyspnea and fatigue (Marcus et al. 1996; Yoshimura et al. 1991;

Mills et al. 1999). Impressively, Colucci et al. found that BNP reduced average pulmonary wedge pressure by 10 mm Hg and increased cardiac index by 400 mL/min per square meter (Colucci et al. 2000).

Perhaps most relevant to this thesis is the positive effects of NPs on the cyclic guanosine monophosphate (cGMP)- protein kinase G (PKG) pathway. Binding of NPs to NPR-A and NPR-B stimulate particulate guanylate cyclase to synthesize cGMP, which activates PKG and leads to positive effects on cardiomyocyte compliance and ultimately, diastolic function (Marso et al. 2016). Such effects will be discussed extensively in subsequent sections.

ANP and BNP are both of cardiac origin, with ANP being primarily synthesized in atrial myocytes and BNP deriving primarily from the ventricles. Notably, ANP and BNP can be released from any chamber of the heart under pathologic conditions (Daniels & Maisel, 2007). ANP is synthesized and stored in granules until atrial distention triggers its release; indeed, ANP is released in response to even minor physiologic circumstances of increased preload, such as exercise. In addition to its diuretic, natriuretic, kaliuretic, and vasodilatory actions, ANP acts as an important counter-regulator to RAAS via its ability to attenuate renin, angiotensin II, aldosterone, and sympathetic nervous system activity (Cuneo et al. 1987; Schultz et al. 1988); thus, in addition to maintaining cardiorenal homeostasis, ANP is critical to blocking acute activation of several of the pathophysiological mechanisms seen in HF.

BNP is directly synthesized *de novo* and secreted from the ventricular myocyte in bursts as pre-proBNP, in response to increased ventricular wall tension resulting from

volume expansion (Pandit et al. 2011). Subsequently, pre-proBNP is cleaved to pro-BNP<sub>1-108</sub> and then further processed to its biologically active form BNP<sub>1-32</sub> and the inactive amino-terminal fragment NTproBNP (Daniels & Maisel, 2007). Given the 1:1 secretion of BNP and NTproBNP, both BNP and NTproBNP can be reliably used in the setting of measuring ventricular dilatation that is seen in the failing heart.

CNP is released primarily from the systemic endothelium in response to shear vascular stress (McFarlane, Winer, & Sowers, 2003) or circulating ANP or BNP (Okahara et al. 1995; Stingo et al. 1992), but can also be synthesized and released by cardiac myocytes (Daniels & Maisel, 2007) and renal glomerular and tubular cells (Mattingly et al. 1994). CNP lacks natriuretic function, instead acting locally via paracrine mechanism to induce vasodilation and inhibit hyperplasia of the vasculature, glomerulus, and interstitial matrix (McFarlane et al., 2003). Moreover, there is evidence that CNP plays a role in long bone growth and development, as studies have shown that CNP-deficient mice exhibit dwarfism and suffer early death compared to their wild-type (WT) counterparts (Chusho et al. 2001).

NPs exert their effects on target tissues via binding and activation of membrane-bound natriuretic peptide receptors (NPRs) that are in turn linked to intracellular second messenger cyclic guanosine monophosphate (cGMP)-dependent signaling cascade (Potter, 2011). As such, cGMP activation upon NP binding is responsible for a cell's physiologic response to NP stimulation. ANP and BNP exert their physiologic effects via Natriuretic Peptide Receptor-A (NPR-A), while CNP action is mediated through NPR-B. NPR-C is responsible for clearing all three NPs from systemic circulation; research has

shown that NPR-C clears 50% of NPs from circulation, while the other half of circulating NPs are enzymatically degraded by NEP (McFarlane et al. 2003). Given both the large physiologic role that NEP has in NP inactivation coupled with the positive cardiovascular effects of NPs, it is perhaps no surprise that NEP inhibition has risen to prominence as an important target in cardiovascular therapeutics. Indeed, NEP inhibition and its effects will be expounded upon further; however, before delving into the role of NEP in HF, it is necessary to briefly delineate HF and its pathophysiology.

### **Heart Failure**

Heart Failure (HF) is a complex clinical condition whereby the heart is incapable of sustaining a Cardiac Output (CO) that is sufficient to meet the metabolic demands of the body's tissues (Kemp & Conte, 2012). Generally speaking, HF arises as a result of structural and functional deficits of the myocardium that results in compromised ejection of blood or ventricular filling (Inamdar & Inamdar, 2016). Such deficits are, on a structural level, result of loss of critical quantity of functional myocytes, often as the result of apoptosis, with subsequent replacement fibrosis of dead myocardial tissue (Kemp & Conte, 2012; Van Heerebeek et al. 2006). Several pathophysiologic mechanisms can induce HF including genetic mutations, ischemic heart disease, hemodynamic overload, ventricular remodeling, interstitial fibrosis, excessive neurohumoral activation, abnormal cardiac myocyte calcium cycling, or myocyte apoptosis (Dassanayaka & Jones, 2015). These mechanisms are not mutually exclusive; instead, many of them can, and often do, occur in concert.

In addition to cardiac dysfunction, it is important to note the relevance of several maladaptive neurohumoral mechanisms that become constitutively activated during HF. Decreased CO in the failing heart results in baroreceptor hypoperfusion, which triggers compensatory activation of the sympathetic nervous system (SNS). Tonicly increased SNS activity combined with renal hypoperfusion leads to activation of RAAS. Initially, these compensatory mechanisms are beneficial: increased SNS activity directly stimulates the heart, increasing CO by means of augmenting heart rate (HR) and stroke volume (SV); simultaneous induction of peripheral vasoconstriction increases mean arterial pressure (MAP), which is further beneficial. RAAS, in addition to its synergistic effects in stimulating peripheral vasoconstriction, induces renal sodium reabsorption that expands extracellular volume (ECV) and increases preload, leading to augmented CO via Frank-Starling mechanism (Kemp & Conte, 2012; Patterson & Adams, 1996).

As previously stated, these maladaptive neurohumoral mechanisms are initially beneficial in improving the cardiovascular and hemodynamic deficits seen in HF. However, with time, chronic neurohumoral activation further hastens the disease process and therefore, is particularly relevant to both understanding and treating the pathophysiology of HF. Tonic SNS activation leads to constitutive activation of adrenergic receptors  $\beta_1$ ,  $\beta_2$ , and  $\alpha_1$  that eventually leads to myocardial toxicity via induction of fetal gene programme, myocyte hypertrophy, and apoptosis (Colucci, 1998). Similarly, tonic RAAS activation also leads to myocyte hypertrophy and interstitial fibrosis (Kim et al. 1995; Konstam et al. 1992). As such, historic HF therapies have largely been directed at attenuating these neurohumoral mechanisms; such therapies will

be further discussed within the framework of NEP inhibition therapy in subsequent sections.

Clinically, HF is typically subdivided into Heart Failure with Reduced Ejection Fraction (HFrEF) and Heart Failure with Preserved Ejection Fraction (HFpEF) based upon measurement of Ejection Fraction (EF), the volumetric fraction of blood ejected from the heart with each heartbeat. If EF is  $\leq 50\%$ , HF is considered HFrEF; conversely, HF with an EF  $\geq 50\%$  is considered HFpEF (Lekavich et al. 2015).

### **Heart Failure with Reduced Ejection Fraction (HFrEF)**

HFrEF is characterized by a markedly decreased capacity of the left ventricle (LV) to supply the requisite amount of oxygenated blood to the rest of the body's tissues that is satisfactory to their metabolic needs. Phenotypically, the LV frequently dilates and acquires an eccentric configuration (Harada et al. 2017), leading to a reduced (or sometimes normal) LV mass/ end-diastolic volume ratio (Inamdar & Inamdar, 2016). HFrEF typically arises as result of pathological mechanisms that lead to large-scale cardiac myocyte loss or dysfunction, such as genetic abnormalities, myocardial infarction (MI), acute myocarditis, or myocardial toxicity (e.g. alcohol, drugs, chemotherapy) (Jessup et al. 2009).

### **Heart Failure with Preserved Ejection Fraction (HFpEF)**

The hallmark of HFpEF is increased LV stiffness such that diastole is impaired and the heart cannot fully relax, leading to poor myocardial compliance and inability of

the LV to fill with blood. Opposite to HFrEF, LV in HFpEF remodels to embody a concentric configuration (Shah, 2013), and has a high LV mass/ end-diastolic volume ratio (Ohtani et al. 2012). LV wall thickness is typically significantly increased compared to both controls and the HFrEF patient cohort (Borlaug & Redfield, 2011). On a microscopic level, this translates to cardiomyocyte hypertrophy with an average increase in size of 50% coupled with interstitial fibrosis (Van Heerebeek et al, 2006). Notably, HFpEF frequently arises as a result of hypertension, aging, and disordered metabolism, which includes dyslipidemia, metabolic syndrome (MetS), and type 2 diabetes mellitus (T2DM) (Packer & Kitzman, 2018).

The relationship between aging and cardiac dysfunction is well established, and there are several biological mechanisms that precede HFpEF often seen in aging: age-related oxidative and mitochondrial stress triggers apoptosis, necrosis, and autophagy that leads to myocyte death. Subsequently, there is induction of compensatory remodeling characterized by biochemical shifts that include increased myofibroblast synthesis and collagen deposition via transforming growth factor  $\beta$ -signaling (Upadhyia & Kitzman, 2017). Furthermore, the equilibrium between matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) becomes perturbed, with downregulation of MMPs and upregulation of TIMPs, ultimately leading to increased extracellular matrix (ECM) and collagen type I accumulation (Weber, Brilla, & Janicki, 1993; Ahmed et al. 2006). Moreover, there is often age-related mitochondrial dysfunction, which on an ultrastructural level, leads to decreased functionality of several key mitochondrial components (Dai & Rabinovitch, 2009; Vermulst et al. 2008;

Vermulst et al. 2007); this is compounded by declining effectiveness of macroautophagy (or mitophagy), the process whereby dysfunctional mitochondria are eliminated (Taneike et al. 2010; Terman et al. 2010).

In sum, these age-related structural and biochemical changes ultimately lead to significant structural remodeling in the heart and vasculature that includes increased LV wall thickness and fibrosis that precedes diastolic dysfunction, and in many people, HFpEF (Chantler & Lakatta, 2012). As such, it is unsurprising that HFpEF is almost exclusively found in older people, particularly women (Upadhyaya et al. 2015), in whom 90% of new HF diagnoses are HFpEF (Gottdiener et al. 2000).

In addition to age-related effects, metabolic disorders such as dyslipidemia, MetS, and T2DM are frequently associated with MetS-related cardiomyopathy that is characterized by myocyte hypertrophy, interstitial fibrosis, and mitochondrial dysfunction (Sverdlov et al. 2016); such dysfunction closely resembles the clinical HFpEF syndrome. Certainly, obesity is a common comorbidity in HF regardless of EF status; this is highlighted by data from the Framingham Heart Study, which showed that, after adjusting for established risk factors, there was a 5% and 7% increase in HF risk for men and women, respectively, for each unit increase in BMI (Prenner & Mather, 2018). Further, once HF begins, BMI is strongly correlated with worse cardiac function and prognosis (Dalos et al. 2016). However, obesity and disordered metabolism are especially associated with HFpEF. The average body mass index (BMI) of HFpEF patients enrolled in clinical trials almost invariably exceeds 25 kg/m<sup>2</sup> and more often exceeds 30 kg/m<sup>2</sup> (Prenner & Mather, 2018). In fact, more than 80% of HFpEF patients

are overweight or obese (Haass et al. 2011). Obesity and associated MetS largely drive HFpEF through their effects on increasing systemic inflammatory mediators that ultimately, promote myocardial stiffness and cardiac dysfunction (Paulus & Tschope, 2013). With increased adiposity, there is pronounced macrophage infiltration of adipose tissue, leading to increased release of pro-inflammatory cytokines (Taube et al. 2012); this theory is corroborated by increased inflammatory markers such as high sensitivity c-reactive protein (CRP) and soluble interleukin 1 receptor-like 1 (ST-2) in HFpEF patients when compared to both HFrEF patients and hypertensive patients (Sanders-van Wijk et al. 2015; Collier et al. 2011). Increased cytokines induce increased myocardial expression of several adhesion proteins such as vascular cellular adhesion molecule (VCAM) and E-selectin that promote migration of leukocytes secreting TGF- $\beta$ , which induces conversion of fibroblasts into myofibroblasts that secrete increased amounts of collagen (Upadhyya & Kitzman, 2017). Ultimately, this leads to profound ECM changes that include enhanced deposition of collagen type I and more collagen cross-linking (Prenner & Mather, 2018). These pathologic changes are directly linked with LV stiffness and diastolic dysfunction characteristic of HFpEF (Westermann et al. 2011; Kasner et al. 2011).

Similarly, it is postulated that systemic inflammation as result of obesity/MetS further contributes to myocardial stiffness and diastolic impairment via disruption of endothelial nitric oxide (NO) release. Normally, NO is a potent stimulator of the cGMP-PKG pathway (Papp et al. 2018); however, depressed NO bioavailability means that the cGMP-PKG pathway is not properly activated (Neal et al. 2017), and the physiologic

process of cGMP-activated PKG phosphorylation of myocardial titin is disrupted. This is significant because PKG phosphorylation of titin is known to lower cardiac myocyte stiffness, promoting myocardial compliance (Marso et al. 2016). Indeed, the decreased cGMP levels and PKG activity seen in obesity/MetS leads to disruption of this physiologic process and is directly associated with increased cardiac myocyte stiffness, leading to the HFpEF phenotype (van Heerebeek et al. 2012). This theory is supported by animal model of HFpEF as well as myocardial biopsy of HFpEF patients, that both showed decreased myocardial cGMP levels and PKG activity (Franssen et al. 2016; Prenner & Mather, 2018).

Diabetes (T2DM) is also a significant risk factor for HFpEF. Similar to the overwhelming incidence of obesity/MetS in HFpEF, there is a very high prevalence of diabetes in HFpEF: data from the CHARM trial showed that among patients with HFpEF, 40% had a diabetes diagnosis, 20% had pre-diabetes, and 22% had undiagnosed diabetes (Papp et al. 2018). This phenomenon is due, in large part, to the same mechanisms that cause HFpEF in MetS. In a ZSF1 diabetic rat model, that also had comorbid obesity and hypertension, there was development of HFpEF secondary to titin hypophosphorylation (Fang, Prins, & Marwick, 2004). Further, there is evidence that increased myocardial inflammation induces formation of reactive oxygen species (ROS) that oxidize and inactivate several biomolecules; notably, diabetics have been shown to have increased oxidation of soluble guanylate cyclase (sGC), cGMP, and PKG, making these molecules unresponsive to the beneficial effects of NO activation (Papp et al. 2018; Upadhyya et al. 2017).

## **Epidemiology**

HF has become increasingly prevalent in modern society, and was deemed an epidemic in 1997 based upon its dramatically increased incidence (Roger, 2013). Overall prevalence for HF in developed countries is estimated to be 1-2% with an annual incidence of 5-10 new cases per 1000 individuals (Mosterd & Hoes, 2007). In the United States alone, there are approximately 5.8 million cases of HF (Roger, 2013) with a yearly incidence of 670,000 cases among US Citizens over 45 years old (Konstantinou, Chatzizisis, & Giannoglou, 2013). A comprehensive review conducted by Lloyd-Jones et al. found that one of every five persons at age 40 in the United States will develop HF at some point in their lifetime (Lloyd-Jones et al. 2010). Despite significant advancements in understanding the pathophysiological underpinnings of HF, prognosis remains poor with five-year and ten-year mortality rates of 50% and 10%, respectively (Macintyre et al. 2000; Mosterd et al. 2001; Cowie et al. 2000).

It is important to note the relevance of both the aging population and the obesity pandemic; currently, there is a greater percentage of people than ever before expected to reach age 80 (Crimmins, 2015), and obesity rates are projected to continue increasing substantially over the next two decades, with an estimated 33% and 130% in obesity and severe obesity prevalence, respectively (Finkelstein et al. 2012). As such, HFpEF has become a particularly salient problem and will continue to have a rising incidence; currently, HFpEF represents half of all HF diagnoses (Yancy et al. 2006) and its prevalence relative to HFrfEF is growing at a rate of 1% per year (Owan et al. 2006). By 2020, HFpEF prevalence is expected to exceed 8% of all people older than 65 years old,

and is expected to greatly increase in younger persons due to the uncontrolled pandemic of obesity (van der Velden, van der Wall, & Paulus, 2016). This growing problem, due largely to the aging population and the “western diet” that has become pervasive in society, highlights the need to better understand the pathophysiology of HFpEF as well as determine novel therapeutic targets for its treatment.

### **Role of Nephilysin in Heart Failure**

NEP has risen to prominence as a target in cardiovascular therapeutics, largely as a result of very promising data showing the effects of NEP inhibitor sacubitril combined with angiotensin receptor blocker (ARB) valsartan for the treatment of HFrEF. This novel 1:1 molar combination of sacubitril/valsartan is referred to in literature as LCZ696, and is marketed by Novartis as Entresto. The PARADIGM-HF (Prospective Comparison of Angiotensin Receptor Nephilysin Inhibitor (ARNi) with ACE Inhibitor (ACEi) to Determine Impact on Global Mortality and Morbidity in Heart Failure) was the largest and most geographically diverse HFrEF trial ever, whereby 8,442 patients with HFrEF (EF  $\leq$  40%, subsequently changed to EF  $\leq$  35% via amended protocol) and New York Heart Association (NYHA) class II-IV were randomized to receive either 200 mg ARNi LCZ696 b.i.d. (4,187 patients) or 10 mg ACEi enalapril (4,212 patients) b.i.d. (McMurray et al. 2013; Bayes-Genis, 2015; Hubers & Brown, 2016). Indeed, the treatment arm receiving LCZ696 had a reduction of 25% in risk of cardiovascular death and 19% in risk a primary endpoint (Solomon et al. 2016). Furthermore, the LCZ696 cohort had a 21% reduction in risk of hospitalization from HF ( $p < 0.001$ ) and

significantly decreased physical limitations of HF symptoms ( $p=0.001$ ) (McMurray et al. 2014). As such, the study was closed early after a median follow-up of 27 months as a result of excess reduced mortality because the boundary for an overwhelming benefit with LCZ696 had been reached (McMurray et al. 2014).

Given the promise of sacubitril/valsartan (LCZ696) in improving clinical outcomes in HFrEF, subsequent studies have sought to see whether said therapy translates to HFpEF. The prospective comparison of ARNi with ARB on management of heart failure with preserved ejection fraction (PARAMOUNT) trial was a randomized, multicenter trial that tested the hypothesis that 200 mg, twice daily LCZ696 is superior to 160 mg, twice daily valsartan with regards to significantly reducing NT-proBNP levels in HFpEF patients (Solomon et al. 2012). Given that NT-proBNP is not a substrate of NEP, it could be reliably measured as a marker of ventricular wall stress even in the presence of NEP inhibition via ARNi. LCZ696 treatment cohort had significantly reduced NT-proBNP levels after 12 weeks but the difference in levels was not sustained at 36 weeks (Huber & Brown, 2016). Translational, cardiac effects of both treatment groups and correlation of NT-proBNP levels to cardiac phenotype were planned to be studied prospectively and such work is currently ongoing; however, of note, left atrial (LA) size significantly decreased in the LCZ treatment arm compared to the valsartan arm, with an average LA volume decrease of 4.6 mL vs. 0.37 mL, respectively (Hubers & Brown, 2016). The decrease in LA dimension is theorized to be a manifestation of reduced hemodynamic stress and improved LV filling pressures in patients receiving LCZ696 (Hubers & Brown, 2016).

Furthermore, Prospective comparison of ARNi with ARB Global Outcomes in heart failure with preserved ejection fraction (PARAGON-HF) trial is another, more extensive multicenter clinical trial investigating the effects of LCZ696 vs. valsartan in improving the composite endpoints of cardiovascular death and HF hospitalizations (Solomon, 2017). PARAGON-HF is currently ongoing and is expected to conclude in May, 2019 (clinicaltrials.gov).

Several studies have suggested the utility of NEP as a novel biomarker in HF prognostication to predict cardiovascular mortality and morbidity; however, definitive, replicated data currently remains sparse. A small trial (n= 350) showed that among patients hospitalized for acute HF, sNEP concentration at admission showed a clear trend towards significance for composite outcomes of HF hospitalizations and death at short-term (2 months) and long-term ( $1.8 \pm 1.2$  years) follow-up (Bayes-Genis et al. 2015). A larger, more encompassing study of 1,069 patients with mainly HFrEF by Bayes-Genis et al. showed that sNEP levels were predictive of CV hospitalization and death (Bayes-Genis, Barallat, Galan et al. 2015). Interestingly, a study of 144 HFpEF patients by Goliash et al. reported that sNEP levels in their HFpEF patient cohort were 3-fold higher than in HFrEF, but showed no significant correlation with prognosis (Goliash, Pavo, Zotter-Tufaro, et al., 2016).

### **The Present Study**

We sought to determine the role that aging and obesity/MetS has on NEP activity. Given that LCZ696 has been so effective at improving outcomes with respect to all

clinical endpoints in HF<sub>r</sub>EF, we were interested to see if these effects translated to HF<sub>p</sub>EF, two common forms of which are caused by aging and MetS/T2DM. Therefore, the goal of this study was to determine whether NEP activity is increased in mice with diastolic dysfunction due to aging or MetS. We hypothesized that both aging and MetS increase NEP activity, contributing to the cardiac dysfunction phenotype seen in the aged and MetS patient populations.

### **SPECIFIC AIMS**

The aims of this thesis are twofold. First, to provide laboratory data in an animal model showing the effect that aging and diet have on NEP activity, for determination as to whether aging and diet-induced obesity increase plasma NEP activity. Second, to determine if LCZ696 can attenuate changes in NEP activity due to aging and diet-induced obesity.

## METHODS

### Animals and Diets

Wild type (WT) C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were fed normal chow diet (NCD) (Advanced Protocol<sup>®</sup> PicoLab<sup>®</sup> Verified 75 IF diet; Lab Diet, St. Louis, MO) for the first two months of their life. At two months of age, mice were switched to either a control diet (CD) (product No. D09071703; Research Diets, Bar Harbor, ME) or high fat, high sucrose diet (HFHS) (product No. D09071703; Research Diets, Bar Harbor, ME) for 4 or 16 months. Several mice (n=4) were not switched to CD or HFHS, but were instead kept on NCD for an additional 4 months to act as an age-matched control against the CD cohort, for determination as to whether CD had a differential effect on NEP activity relative to NCD.

CD consisted of 10% kcal lard and 0% sucrose; HFHS consisted of 58% kcal lard and 13% kcal sucrose. Diets were matched by nutrient/caloric value; full diet composition is provided in table 2. Simplified breakdown of NCD, CD, and HFHS diets by macronutrient calorie (kcal%) is provided in table 3. Mice were housed four per cage, with a 12 hour light/12 hour dark cycle. Both food and water were provided ad libitum. The protocol was approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Product #	D09071702		D09071703	
	gm%	kcal%	gm%	kcal%
Protein	20.5	15	15.0	15
Carbohydrate	38.2	28	76.3	75
Fat	35.5	58	4.5	10
Total		100.0		100.0
kcal/gm	5.54		4.05	
Ingredient	gm	kcal	gm	kcal
Casein	182	728	182	728
DL-Methionine	3	12	3	12
Maltodextrin 10	170	680	170	680
Corn starch	0	0	760	3040
Sucrose	164	656	0	0
Lard	320	2880	55	495
Salt Mix, S10026B	50	0	50	0
Vitamin Mix, V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Total	901	4996	1232.0	4995

**Table 2. Control Diet (CD) and high fat, high sugar (HFHS) diet composition.**  
D09071702 is HFHS diet and D09071703 is CD diet (Research Diets, Bar Harbor, ME).

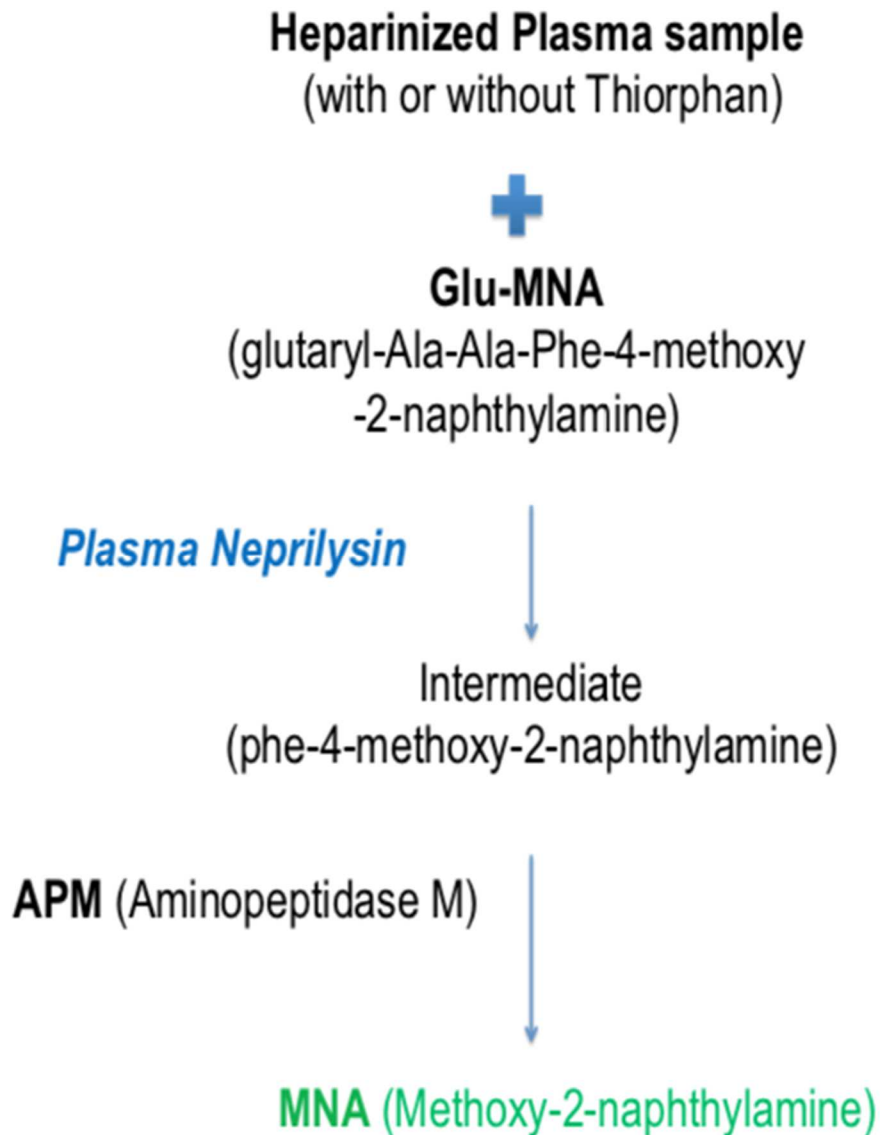
<b>% kcal</b>	<b>Normal Chow Diet (NCD)</b>	<b>Control Diet (CD)</b>	<b>High Fat High Sucrose (HFHS)</b>
<b>Protein</b>	<b>23</b>	<b>15</b>	<b>15</b>
<b>Fat</b>	<b>13 Soybean Oil</b>	<b>10 Lard</b>	<b>58 Lard</b>
<b>Carbohydrate</b>	<b>64 Mostly Starch</b>	<b>75 Corn Starch</b>	<b>28 Sucrose</b>

**Table 3. Diet composition and calorie (kcal%) breakdown.** NCD is Advanced Protocol<sup>®</sup> PicoLab<sup>®</sup> Verified 75 IF diet; Lab Diet, St. Louis, MO; CD is product no. D09071703; HFHS is product no. D09071702 (Research Diets, Bar Harbor, ME)

### **Neprilysin activity assays**

Neprilysin activity assays were performed on plasma isolated at baseline from 2 month old mice fed NCD (BL), and again from mice fed CD and HFHS diet following 4 and 16 months of feeding. NEP activity was also assayed in mice that remained on NCD for 4 additional months, being fed NCD for a total of 6 months. Mice were anesthetized by placement in a bell jar chamber that contained isoflurane moistened paper, and incision was then made on the distal tail. Heparinized capillary action blood tubes (Sigma Aldrich, St. Louis, MO) were then used to collect blood. All samples were collected between 9 AM and noon. Collected blood samples were centrifuged to obtain plasma, which was then stored at  $-80^{\circ}$  C, without previous freeze-thaw cycles, prior to assay.

Neprilysin activity was measured via adapted protocol from Willard et al. (Willard et al. 2017). In the neprilysin activity assay, plasma samples were incubated with glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamine (Glu-MNA) and Aminopeptidase M (APM). Each plasma sample was assayed in both the presence and absence of the specific NEP inhibitor, DL-thiorphan, to distinguish plasma NEP activity from non-specific endopeptidase activity. Glu-MNA was broken down by plasma NEP (sNEP) into the intermediate, phe-4-methoxy-2-naphthylamine. APM then degraded intermediate phe-4-methoxy-2-naphthylamine into the fluorescent product, methoxy-2-naphthylamine (MNA), which was read via fluorescent plate reader and compared against an MNA standard curve. A diagram of the neprilysin activity assay can be seen in figure 1.



**Figure 1. Neprilysin Activity Assay.** Outline of NEP activity assay (adapted from Willard et al.) is shown, with relevant enzymes and substrates.

First, reagents were prepared to their final working concentrations. Stock solution of 50 mM tris-HCl buffer was made. MNA (Sigma-Aldrich, St. Louis, MO) was dissolved in DMF (dimethyl formamide) to 10 mM, then diluted with tris-HCl buffer to 1  $\mu$ M and stored at -20° C. Glu-MNA (Sigma-Aldrich, St. Louis, MO) was dissolved in DMF to 10 mM and stored at -20° C until the day of the assay, whereby it was diluted with tris-HCl buffer to 1 mM. DL-thiorphan (Sigma-Aldrich, St. Louis, MO) was dissolved in 100% ethanol to 10 mM, then diluted with tris-HCl buffer to 1 mM and stored at -20° C until the day of the assay, whereby it was diluted with tris-HCl buffer to 25  $\mu$ M. Dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO) was diluted in dH<sub>2</sub>O to 11 mM and stored at -20° C. APM (R & D Systems, Minneapolis, MN) was diluted with tris-HCl buffer to final concentration of 1  $\mu$ g/10 $\mu$ L.

Neprilysin activity assay was conducted using a 96-well, half-area plate (Sigma-Aldrich, St. Louis, MO). Each plasma sample was assayed in triplicate, both in the presence and absence of DL-thiorphan. Sample wells with added DL-thiorphan received 3  $\mu$ L of DL-thiorphan, 1  $\mu$ L of APM, 14  $\mu$ L tris-HCl buffer, and 5  $\mu$ L plasma. Sample wells without DL-thiorphan received 1  $\mu$ L APM, 17  $\mu$ L tris-HCl buffer, and 5  $\mu$ L plasma. 96-well, half-area plate was then covered with sealing tape and placed on a plate shaker at 37° C for 10 minutes. Subsequently, 2  $\mu$ L Glu-MNA was added to all sample wells to achieve 80  $\mu$ M final concentration. Plate was recovered with sealing tape and put back on the plate shaker at 37° C for 30 minutes. MNA standard curve was then created: MNA was diluted in tris-HCl buffer to create 100  $\mu$ M MNA working stock. 100  $\mu$ M MNA working stock was then added to an eppendorf tube in the following amounts to

create each MNA standard: 0  $\mu\text{L}$ , 1.25  $\mu\text{L}$ , 2.5  $\mu\text{L}$ , 5  $\mu\text{L}$ , 10  $\mu\text{L}$ , 20  $\mu\text{L}$ , 30  $\mu\text{L}$ , and 60  $\mu\text{L}$ . All tubes were then filled with the corresponding volume tris-HCl buffer to create 500  $\mu\text{L}$  total volume. This corresponded to 0, 6.25, 12.5, 25, 50, 100, 150, and 300 pmol MNA. 25  $\mu\text{L}$  of each MNA standard was added in duplicate to the 96-well, half-area plate. 3.1  $\mu\text{L}$  DTT was added to all wells containing either sample or standard. Finally, fluorescence was read via fluorescent plate reader (Tecan Infinite® M1000 Pro Microplate Reader) with excitation wavelength of 340 nm and emission wavelength of 420 nm. Triplicate results were averaged and samples incubated with DL-thiorphan were subtracted from samples incubated without DL-thiorphan to give plasma NEP activity. Results were compared against the MNA standard curve, and expressed as pmol MNA/hour/ $\mu\text{L}$  plasma.

### **Treatment with LCZ696**

Prior to LCZ696 treatment, plasma was isolated and frozen from mice fed CD and HFHS diet for 16 months, with accordance to the protocol outlined above. Mice were then treated with 100 mg/kg LCZ696 for one week. Mice water consumption was measured (figure 2) for approximation of LCZ696 concentration to put in water to achieve desired 100 mg/kg dose. LCZ696 was then solubilized in drinking water and added to their cages for consumption ad libitum. Immediately following one week of LCZ696 therapy, plasma was once again isolated according to the protocol outlined above. NEP activity assay was performed on plasma samples from mice pre- and post-treatment with 100 mg/kg LCZ696.

## **Statistical Analyses**

Comparison among diet groups: BL, NCD, CD, and HFHS was accomplished via one-way analysis of variance (ANOVA). On several occasions, direct comparison between two groups was considered more appropriate and was performed via unpaired, non-parametric t-test (mann-whitney test). P values < 0.05 were considered significant.

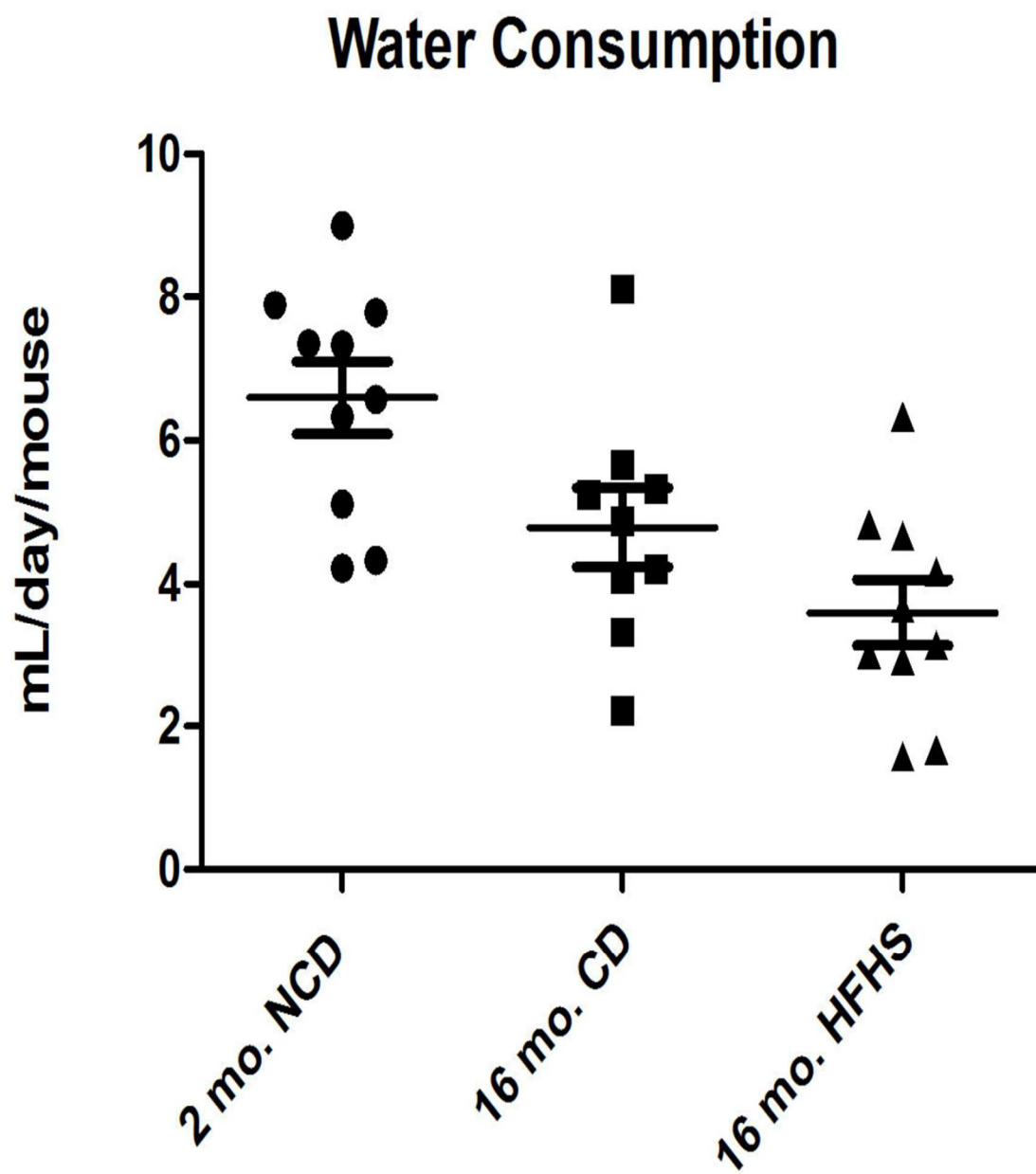
## RESULTS

As previously mentioned, NEP activity was measured and compared against an MNA standard curve, an example of which is shown in figure 3 below. Baseline NEP activity (BL) was derived from mice fed Normal Chow Diet (NCD) for 2 months and averaged  $5.0 \pm 2.9$  pmol MNA/hour/ $\mu$ L plasma. Measurement of whether NEP activity was changed in the cohort that was not switched to CD or HFHS, but was instead kept on NCD for an additional 4 months to act as the age-matched control against the CD cohort was performed: mice that remained on NCD for 4 additional months had NEP activity of  $4.67 \pm 0.7$  pmol MNA/hour/ $\mu$ L plasma ( $p$ =ns vs. BL), whereas mice switched to CD for 4 months following BL had NEP activity of  $14.8 \pm 6.4$  pmol MNA/hour/ $\mu$ L plasma ( $p$ <0.0002 vs. BL). For this reason, mice were fed CD for all subsequent experiments. These data are shown in figure 4.

After being fed NCD for the first 2 months of their life (BL), mice were switched to either CD or HFHS diet for 4 or 16 months, which allowed for determination of the effects of both age and diet on NEP activity. NEP activity in mice fed CD for 4 months was  $14.8 \pm 6.4$  pmol MNA/hour/ $\mu$ L plasma and was further increased at 16 months on CD:  $65.3 \pm 16.4$  pmol MNA/hour/ $\mu$ L plasma ( $p$ =0.0028). NEP activity in mice fed HFHS for 4 months was  $20.0 \pm 7.6$  pmol MNA/hour/ $\mu$ L plasma and was further increased at 16 months on HFHS diet:  $43.8 \pm 20.9$  pmol MNA/hour/ $\mu$ L plasma ( $p$ =0.0031). These results clearly highlight the powerful effect of age on NEP activity. Comparison of the age-matched effect of HFHS diet relative to CD via unpaired non-parametric t-test (mann-whitney test) revealed that there was a trend towards significance

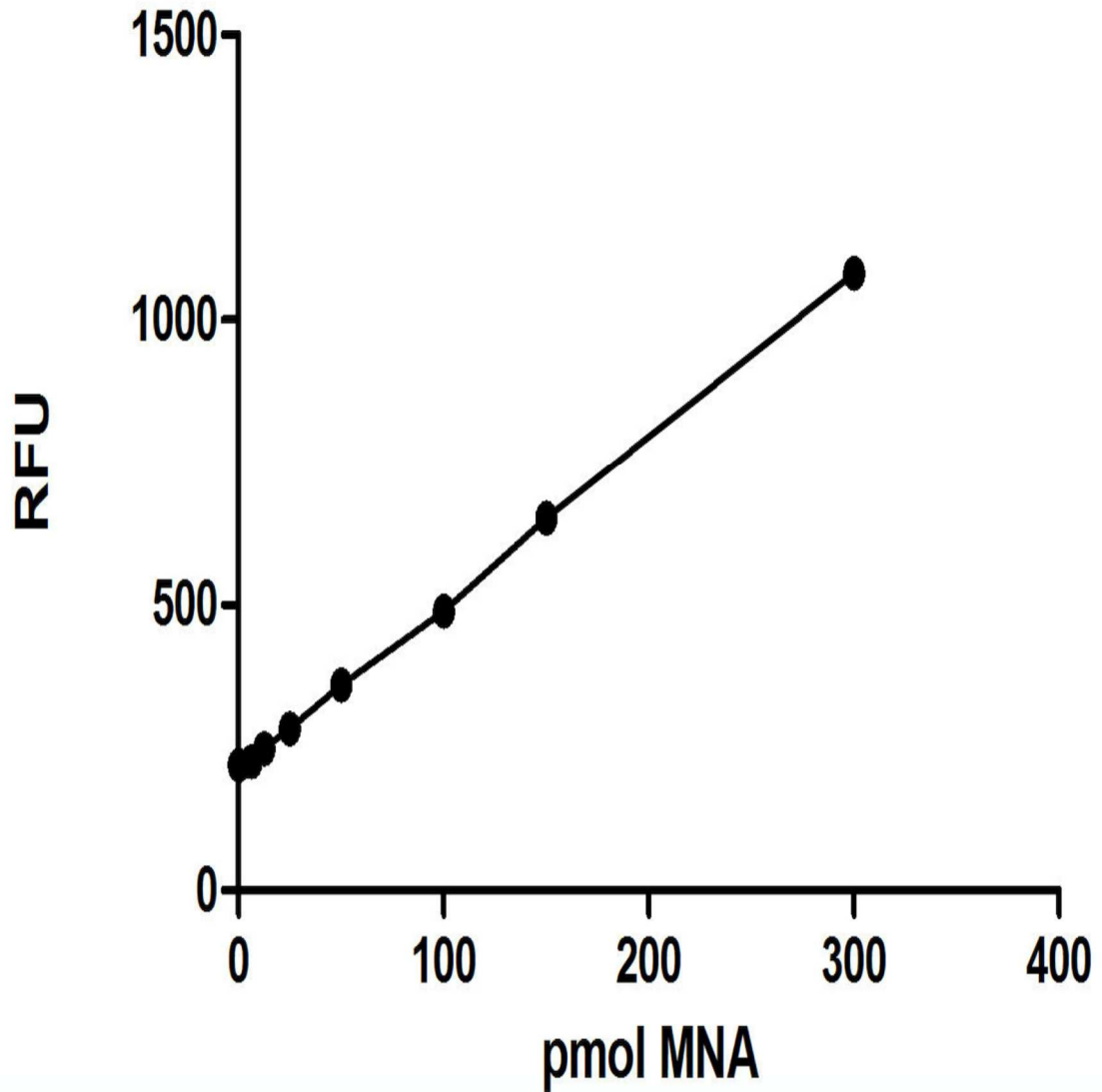
at 4 months ( $p=0.06$  vs. CD) but not at 16 months ( $p=ns$  vs. CD). These data are shown in figure 5.

Mice fed CD and HFHS for 16 months that received one week of 100 mg/kg LCZ696 therapy had 78% and 85% inhibition of their plasma NEP (sNEP) activity, respectively. Prior to treatment with 100 mg/kg LCZ696, CD cohort had average NEP activity of  $65.3 \pm 16.4$  pmol MNA/hour/ $\mu$ L plasma; average NEP activity in this cohort decreased to  $14.7 \pm 9.6$  pmol MNA/hour/ $\mu$ L plasma following one week of 100 mg/kg LCZ696 therapy (78% inhibition;  $p= 0.0286$ ). HFHS cohort had average NEP activity of  $43.8 \pm 20.9$  pmol MNA/hour/ $\mu$ L plasma prior to treatment with 100 mg/kg LCZ696; this decreased to  $6.4 \pm 4.6$  pmol MNA/hour/ $\mu$ L plasma immediately following one week 100 mg/kg LCZ696 treatment (85% inhibition;  $p= 0.0006$ ). Matched NEP activity values for individual mice pre- and post-treatment with 100 mg/kg LCZ696 are shown in figures 6 and 7.



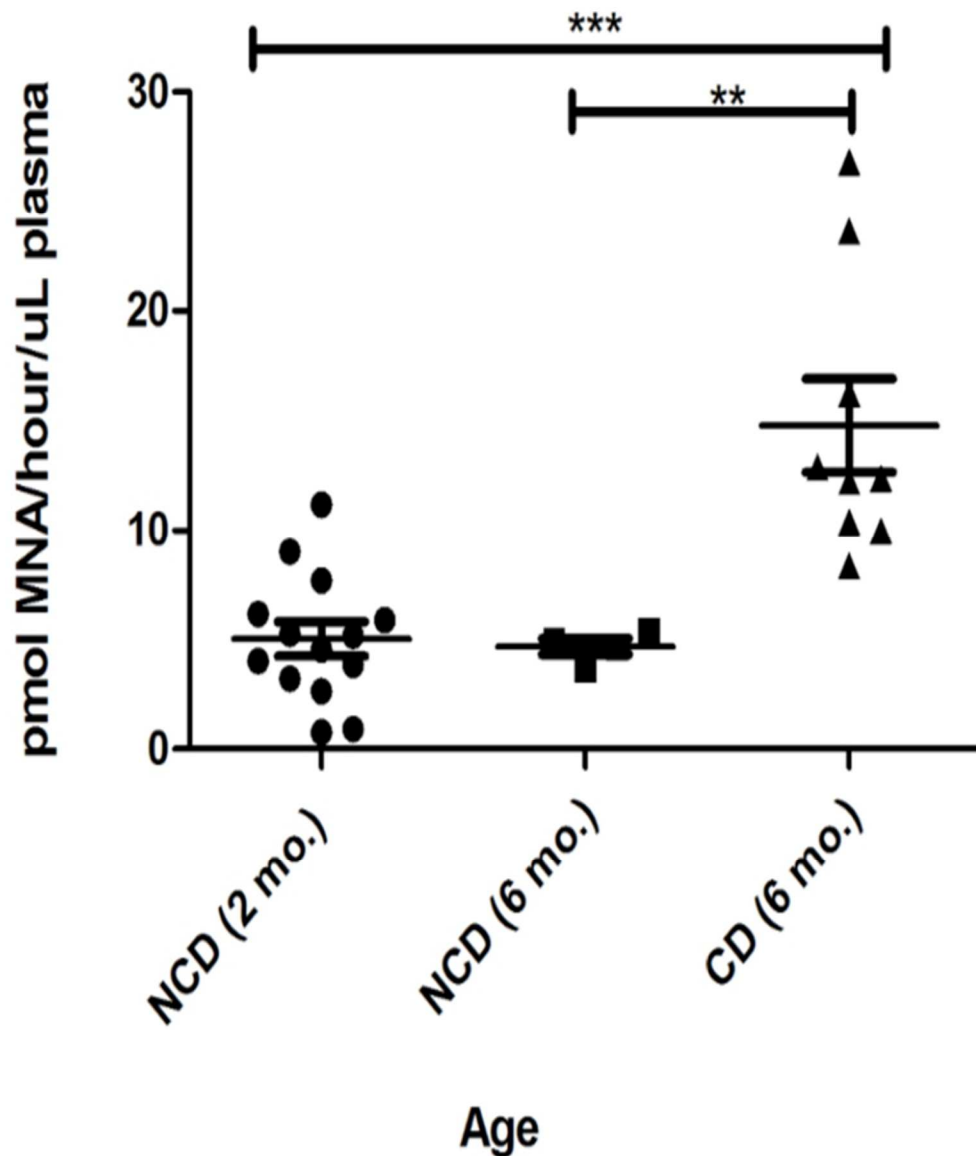
**Figure 2. Water Consumption in Aged mice.** Water consumption in the CD and HFHS diet cohorts was measured to best approximate the concentration of LCZ696 to add, such that the desired dosage of 100 mg/kg LCZ696 would be achieved.

## MNA Standard Curve



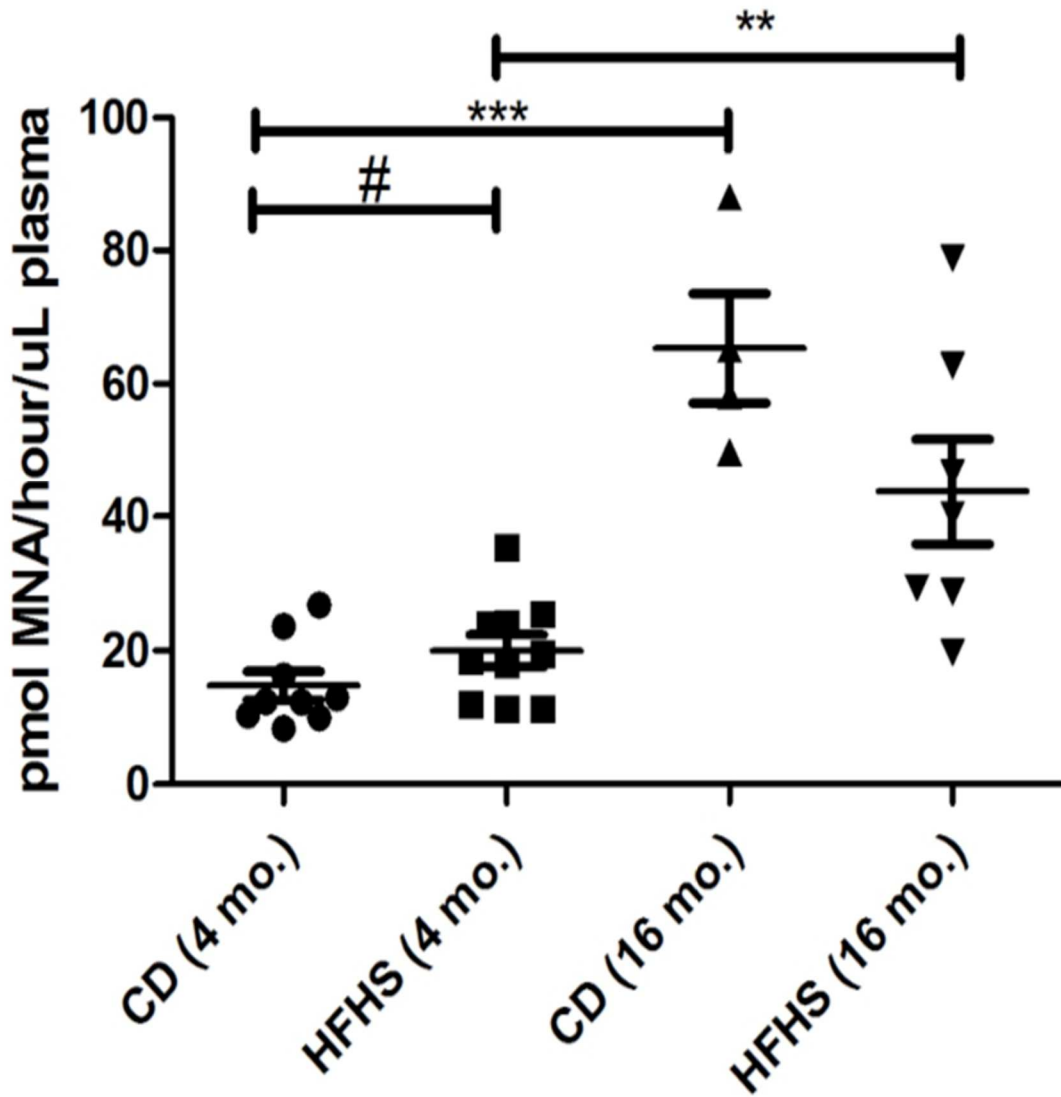
**Figure 3. MNA standard curve.** MNA standard curve was generated and utilized for determination of NEP activity in plasma samples with unknown NEP activity.  $r^2$  values < 0.98 were not accepted.

## Normal Chow Diet (NCD) vs. Control Diet (CD)



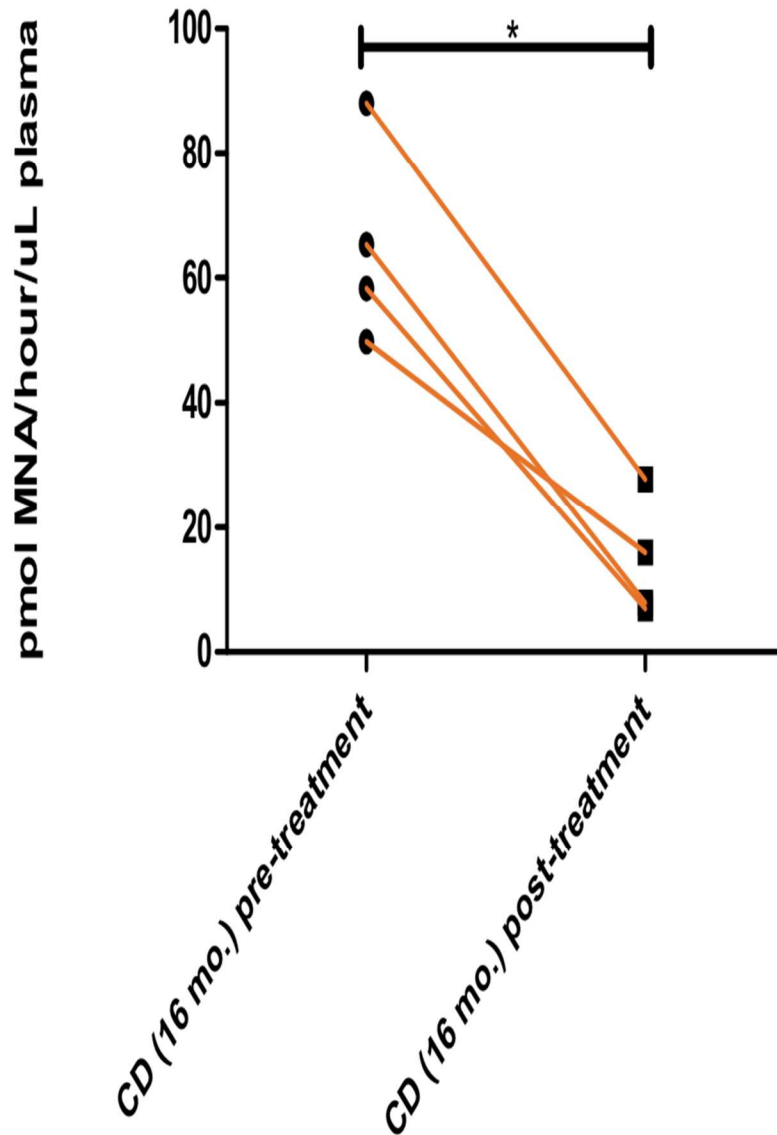
**Figure 4. Control Diet (CD) increases NEP activity.** Baseline NEP activity (BL,  $5.0 \pm 2.9$ ) was derived from mice fed Normal Chow Diet (NCD) for 2 months. NEP activity was not significantly increased in the NCD cohort at 6 months ( $4.67 \pm 0.72$ ;  $p = \text{ns}$  vs. BL); after 2 months on NCD, mice were switched to CD for 4 months and exhibited increased plasma NEP activity ( $14.8 \pm 6.4$ ;  $p < 0.0002$  vs. BL). For this reason, mice were fed CD for subsequent experiments.

## Aging & Metabolic Syndrome



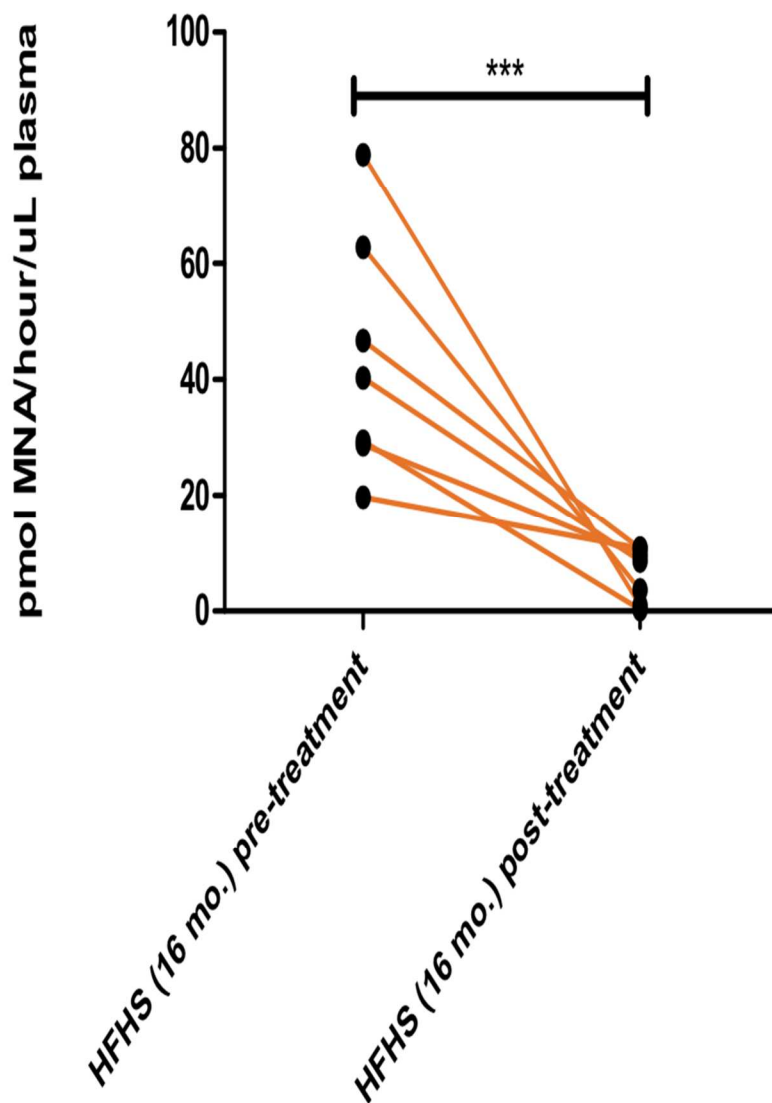
**Figure 5. Aging exerts a greater influence on NEP activity than Metabolic Syndrome (MetS).** Mice fed HFHS diet for 4 or 16 months develop a MetS phenotype. Compared to mice fed CD, NEP activity trended towards an increase in mice fed HFHS at 4 months ( $20.0 \pm 7.6$ ;  $p=0.06$  vs. CD), but not at 16 months ( $43.8 \pm 20.9$ ;  $p=ns$  vs. CD). NEP activity in CD-fed mice increased from 4 months to 16 months ( $14.8 \pm 6.4$  vs.  $65.3 \pm 16.4$ ;  $p=0.0028$ ). NEP activity in HFHS-fed mice increased from 4 months to 16 months ( $20.0 \pm 7.6$  vs.  $43.8 \pm 20.9$ ;  $p=0.0031$ )

## Neprilysin activity pre- and post-treatment with LCZ 100 mg/kg



**Figure 6. One week of treatment with 100 mg/kg LCZ696 significantly decreased plasma NEP activity in mice fed CD for 16 months.** NEP activity was measured in each mouse pre- and post-treatment with 100 mg/kg LCZ696. Mice fed control diet (CD) for 16 months had elevated NEP activity compared to both BL and mice fed CD for 4 months that was significantly decreased following one week of 100 mg/kg LCZ696 therapy.

## Neprilysin activity pre- and post-treatment with LCZ 100 mg/kg



**Figure 7. One week of treatment with 100 mg/kg LCZ696 significantly decreased plasma NEP activity in mice fed HFHS for 16 months.** NEP activity was measured in each mouse pre- and post-treatment with 100 mg/kg LCZ696. Mice fed high fat, high sucrose diet (HFHS) for 16 months had elevated NEP activity compared to both BL and mice fed HFHS diet for 4 months that was significantly decreased following one week of 100 mg/kg LCZ696 therapy.

## DISCUSSION

To our knowledge, this study is the first to have directly examined the effects of both aging and diet-induced MetS on plasma NEP activity. This research demonstrated that NEP activity increases with aging, and that aging exerts a greater influence on NEP activity than MetS does. Aging exerted a powerful effect on NEP activity, regardless of diet composition. NEP activity in CD-fed mice increased from 4 months to 16 months on diet ( $14.8 \pm 6.4$  vs.  $65.3 \pm 16.4$ ;  $p=0.0028$ ), and NEP activity in HFHS-fed mice also increased from 4 months to 16 months on diet ( $20.0 \pm 7.6$  vs.  $43.8 \pm 20.9$ ;  $p=0.0031$ ). An interesting finding of this study was that mice fed CD for 6 months had increased plasma NEP activity ( $14.8 \pm 6.4$ ) compared to mice fed NCD for the same period ( $4.67 \pm 0.72$ ). It was then shown that both CD and HFHS increased NEP activity compared to NCD. Examination of the age-matched effect of HFHS diet relative to CD revealed a trend towards significance at 4 months ( $20.7 \pm 7.6$ ;  $p=0.06$  vs. CD) but not at 16 months ( $43.8 \pm 20.9$ ;  $p=ns$  vs. CD). Importantly, it was shown that LCZ696 can effectively attenuate plasma NEP activity. Mice fed CD and HFHS diet for 16 months that received one week of 100 mg/kg LCZ696 therapy had 78% inhibition ( $p=0.0286$ ) and 85% inhibition ( $p=0.0006$ ) of their plasma NEP activity, respectively.

### Effect of Aging

The present study found a clear relationship between aging and NEP activity, regardless of diet composition. Mice in both the HFHS and CD-fed cohorts exhibited significantly increased plasma NEP activity as they progressed from 4 months on diet to

16 months on diet. No prior study to date has directly examined the relationship between aging and plasma NEP activity, and certainly more work needs to be done in a human population; however, these results suggest that the increased NEP activity seen in aging could contribute, at least in part, to the cardiac dysfunction and HFpEF phenotype seen in aging. Indeed, the effect of increased NEP activity is deleterious to the heart primarily as a result of its effect on augmenting NP degradation. Given that NPs stimulate cGMP synthesis (Piggott et al. 2006), it is plausible to conclude that NP deficiency leads to reduced cGMP activation, which limits activation of the myocardial cGMP-PKG pathway. Attenuated PKG activity leads to hypophosphorylation of titin at the N2B unique sequence, which causes increased cardiomyocyte stiffness, reduced compliance, and contributes to the HFpEF phenotype (Papp et al. 2018). This theory is supported by a pioneering study from Franssen et al. showing that myocardial biopsy obtained from HFpEF patients exhibited reduced myocardial cGMP content and PKG activity (Franssen et al. 2016). Further, experiments of short-term BNP administration and cGMP-enhancing treatment with sildenafil in a large animal model have showed enhancement of PKG-mediated titin phosphorylation, and resulted in improvement of diastolic function (Zinman et al. 2015).

### **Effect of CD diet**

An unexpected, but nonetheless important discovery in this study was that CD had a greater effect on NEP activity than NCD did. Upon investigation into the composition of each diet (figure 3), it was determined that while they are roughly

equivalent in terms of caloric breakdown by macronutrient, NCD fat source (13% kcal) derives from soybean oil whereas CD fat (10% kcal) derives from lard. Given that only 14% of the fatty acids in soybean oil are saturated, soybean oil is a largely unsaturated fat source; in contrast, lard is 40% saturated fat, making it a much more concentrated source of saturated fat (Gunstone 1996). The adverse effects of saturated fats on cardiometabolic health is well documented (Sacks et al. 2017), and a recent study by Zraika et al. found that prolonged exposure to the saturated fatty acid (FA) palmitate upregulated islet NEP activity, leading to reduced insulin pre-mRNA and ATP levels, oxidative/nitrative stress, attenuated potassium and calcium channel activities, and decreased glucose-stimulated insulin secretion (Zraika et al. 2013). However, no research to date has examined the differential effects of saturated vs. unsaturated fat on plasma NEP (sNEP) activity, and it remains plausible that the deleterious effects of saturated fat on the heart are due, at least in part, to its effect on increasing plasma NEP activity. Certainly, this mandates further experimentation, as this study was not set up to determine such data.

### **Effect of HFHS diet**

In this study, there was a lack of congruency in the results with regards to the role of obesity-related MetS on plasma NEP activity. NEP activity in mice fed HFHS diet for 4 months showed a trend towards significance ( $p=0.06$ ) compared to the CD-fed mice at 4 months, but that trend was not present at 16 months. However, both HFHS and CD-fed mice exhibited increased NEP activity compared to mice fed NCD, perhaps as a result of

the increased saturated fat content in those diets compared to NCD. Interestingly, a 2011 study by Standeven et al. found that in humans, plasma NEP concentrations were increased in patients with MetS. Their study, which examined 318 white European males, found that MetS patients had elevated circulating plasma NEP levels, and increased progressively with degree of insulin resistance, body mass index (BMI), and increasing MetS components (Standeven et al. 2011). Similarly, a study by Rice et al. also showed that plasma NEP concentration correlated significantly with BMI as well as with cholesterol, and triglycerides (Rice et al. 2006). A third very compelling study measured circulating NEP levels in bariatric (Roux-en-Y gastric bypass) surgery patients pre- and post-operation, and found that plasma NEP fell  $27\pm 16\%$  following bariatric surgery, further reinforcing the probable notion that obesity-related MetS affects NEP activity in some capacity (Ghanim et al. 2018).

Ultimately, the amalgamation of these results highlight the need for further work to be done to fully elucidate the role of diet, and specifically MetS, on NEP. As previously stated, HFHS has an effect, and saturated fat may have a profound effect on plasma NEP activity. If future work does find a more definitive link between MetS and NEP activity, it could help explain the phenomenon of low NP levels in obese patients compared to their normal weight counterparts despite having a similar degree of clinical HF (Prenner & Mather, 2018). This phenomenon has traditionally been explained by the high concentration of the receptor that clears NPs from circulation, NPR-C, in adipose tissue coupled with the increased amounts of adipose tissue in obese patients, which ostensibly directly decreases circulating NP levels (Wang et al. 2004). However, if a

clear link is established between increased plasma NEP activity in MetS patients, it could help mechanistically explain this phenomenon more completely.

### **Inhibition by LCZ696**

There have been several prominent trials, notably PARADIGM-HF, that have established the effectiveness of LCZ696 in improving clinical outcomes in HFrEF patients. However, to date, there has been very little research on LCZ696 as a therapy for HFpEF. Given the established causal relationship of both aging and diet-induced obesity with HFpEF, this research is fundamental to better understanding and characterizing the role of plasma NEP activity in the pathogenesis of HFpEF. Importantly, a central finding of this study was that LCZ696 could effectively attenuate the elevated plasma NEP activity seen in the aged mice fed CD (78% inhibition;  $p=0.0286$ ) and HFHS diet (85% inhibition,  $p=0.0006$ ) for 16 months. As such, using LCZ696 therapy in this patient cohort to decrease pathologically elevated NEP activity will lead to augmentation of NP levels (Bayes-Genis, Barallat, & Richards, 2016). This will result in increased activation of the myocardial cGMP-PKG pathway, ultimately culminating in increased titin phosphorylation and improvements in cardiomyocyte compliance and ostensibly, the HFpEF phenotype.

### **Limitations of the Study**

As previously mentioned, this study was not set up to determine the differential effects of saturated fat vs. unsaturated fat on plasma NEP activity. While we

hypothesized that CD and HFHS diet increased plasma NEP activity as a result of the increased saturated fat content in those diets relative to NCD, it is entirely possible that there were other confounding variables present that contributed to this result. As such, the cornerstone of future work should focus on manipulating saturated fat content as the independent variable, with focus on how differential quantitation of fatty acid saturation in food contributes to both plasma NEP activity and diastolic function.

Moreover, it should be noted that while several studies have examined plasma NEP levels, mostly in the context of MetS patients (Standeven et al., 2011; Rice et al., 2006; Ghanim et al. 2018), very few studies to date have examined plasma NEP activity in any capacity. Therefore, future work within this area of study should measure both circulating plasma NEP levels and plasma NEP activity as a means to better understand the relationship between the two. Finally, and perhaps most importantly, future research should investigate plasma NEP activity in human populations with both MetS and aging, and directly correlate NEP activity to cardiac health and diastolic function, using clinical parameters.

### **Clinical Implications**

The beneficial effects of NP-mediated activation of the myocardial cGMP-PKG pathway on diastolic function highlight the relevance and promise of LCZ696 as a therapy for HFpEF patients. Treatment with NEP inhibitors in healthy humans as well as HF patients has been shown to reliably and sustainably increase plasma NP concentrations as well as circulating and urine cGMP levels (Bayes-Genis, Barallat, &

Richards, 2016). Honing and optimizing a therapy that can increase NP and cGMP levels via inhibition of NEP activity appears to be a very promising treatment option, especially in aged and MetS human patient populations that potentially exhibit significantly elevated plasma NEP activity; this sentiment is especially true in the age of precision medicine, whereby plasma NEP activity can be measured before decision as to whether the patient would benefit from NEP inhibition therapy. Ultimately, data from this study suggest that aging exerts a powerful effect on NEP activity and diet appears to have an effect as well, albeit smaller and less well defined; certainly, more work needs to be done in both animal model and human populations to definitively elucidate the role of both aging and MetS on NEP activity. Finally, to reiterate, LCZ696 remains an interesting, promising, and possibly very effective therapy for aging and diet-induced HFpEF, a growing and largely fatal disease that, thus far, has been defined by lack of effective therapies.

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**CURRICULUM VITAE**

