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EFFECTS OF THE NA-CL CO-TRANSPORTER (NCC) IN WESTERN DIET INDUCED METABOLIC AND CARDIAC DYSFUNCTION

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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Abstract

EFFECTS OF THE NA-CL CO-TRANSPORTER (NCC) IN WESTERN DIET INDUCED METABOLIC AND CARDIAC DYSFUNCTION

Zachary S. Cutter

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2018.

Major Director: STEFANO TOLDO, PH.D. Assistant Professor Department of Internal Medicine, Division of Cardiology, Department of Physiology and Biophysics

Obesity and heart failure are increasingly known as pro-inflammatory states that carry a significant and debilitating health burden. High-fat diets represent a common pathogenic route to obesity and future development of heart failure. Interleukin-18, a pro-inflammatory cytokine, has been identified as essential for metabolic and glucose homeostasis; however also mediates cardiac dysfunction, in particular diastolic dysfunction. A divergence of mouse IL-18KO and IL-18RKO metabolic phenotypes have been shown and, additionally, the Na-Cl Co-transporter (NCC) has recently been identified as a novel receptor for IL-18. Therefore, we hypothesized that NCC mediates the IL-18 induced positive metabolic and negative cardiac effects. Using male C57BL/6J mice, we evaluated metabolic and cardiac function changes in wild-type, IL-18KO, and NCCKO mice after at least 8 weeks of high saturated-fat and high sugar diet (Western Diet, WD). This was accomplished by measurements of body weight gain, fasting glucose, and cardiac systolic and diastolic function parameters. We report NCCKO mice had a significantly increased body weight gain compared to baseline vs. wild-type mice (55% vs. 22%). Additionally, NCCKO mice had a lower percent worsening from baseline in isovolumetric relaxation time (IRT) (29% vs. 123%) and Tei Index (62% vs. 162%) vs. wild-type mice ($P<0.05$). Collectively, the metabolic and cardiac changes in NCCKO mice after WD resembled those of IL-18KO mice on the same diet. The Na-Cl Co-transporter may function to mediate metabolic and cardiac effects of IL-18 during high-fat diet feeding and its possible role in doing so warrants further investigation.

Introduction:

Obesity: Prevalence, Classification, and Health Risks

Combinations of poor quality diet, sedentary lifestyle, smoking, and socioeconomic status continue to cause and be associated with the frightening prevalence of obesity, where 37.7% of adults and 17.2% of youth in the United States were obese (BMI ≥30) in 2014 (1,2). Although burdened by its limitations in finely discriminating between body fat % and lean mass (3,4), body mass index, (BMI, calculated as weight in kilograms divided by height in meters squared) for the time being remains the conventional measure used in broad population studies to evaluate body composition due to its ease in obtaining and reproducibility. Before understanding potential health implications of obesity, it is important to define the spectrum of BMI classifications. As indicated in Table 1, the calculated value of a patient's BMI stratifies them into defined groupings where increases in BMI are labeled as worsened obesity.

Table 1. BMI classifications

Body Mass Index (BMI) classification

	Underweight	***Normal***	***Overweight***	***Obese***	***Class II Obesity***	***Class III Obesity***
kg/m^2	<18.5	18.5-24.9	25-29.9	30-34.9	35-39.9	>40

Depicted in Figure 1, a J or U-shaped curve exists correlating BMI to risk of mortality; where a BMI classification that is either lower than normal or obese carries with it an increased *risk* of all-cause mortality ([5, 6]). The risk for cardiovascular diseases also exhibit similar trends with BMI. When stratified into normal, overweight, or obese BMI it is apparent that obesity is associated with higher *risk* of future development of heart failure. In fact, for each 1 increment increase in BMI, the *risk* of heart failure increases 5% for men and 7% for women ([7]). This evidence clearly places obesity (BMI>30 kgm^{-2}) as a risk factor for development of heart failure.

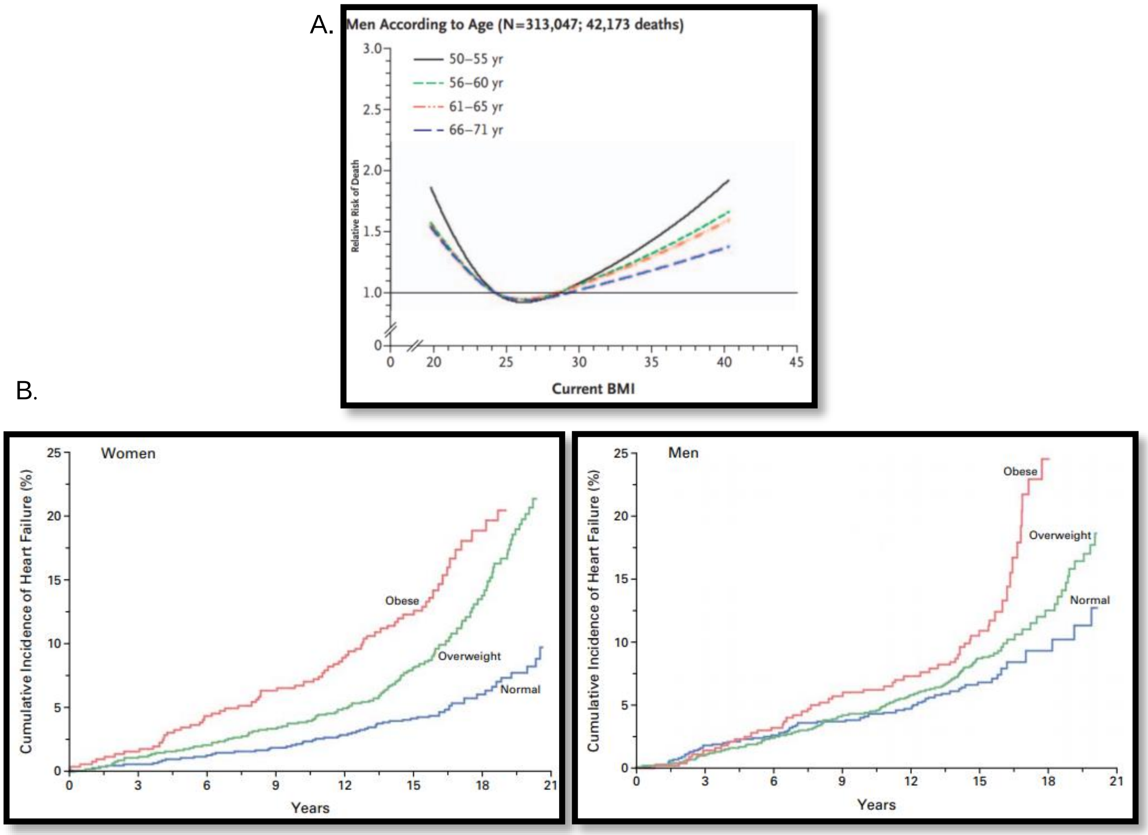

Figure 1. Heart Failure Incidence with Obesity: Adapted from Adams et al, NEJM, 2006. Relationship of BMI to relative risk of death compared to the reference group (BMI 23.5-24.9) with respect to age groups (A). Adapted from Kenchaiah et al, NEJM, 2002. Increase incidence of heart failure with increasing BMI in aging women and men (B).

Heart Failure: Definitions, pathophysiology, and paradoxical prognosis with obesity

Normally, the heart and the vasculature are able to adapt and supply adequate amounts of oxygenated blood to match increased physiological demands of the body. When the heart is unable to supply sufficient oxygenated blood to match systemic needs, whether at baseline or during exertion, and symptoms of shortness of breath, dyspnea on exertion, fatigue, or exercise intolerance become clinically apparent, a patient is diagnosed with the syndrome of heart failure. Echocardiographic assessment of cardiac function is essential to evaluating a patient's status, future prognosis, and tracking of medical management. Ejection Fraction (EF, %) is a calculated value derived from the difference in volume estimations at end diastole (EDV) and end systole (ESV) of the cardiac cycle ([EDV-ESV]/EDV]*100) that is used to evaluate aspects of cardiac contractile function. Population based studies have helped establish normal reference values for EF where guidelines refer to a normal EF for an adult to be 62% with a 2 standard-deviation range to be 52-72% (8).

While proper contractile function of the heart is undeniably essential, the ability of the heart to relax and properly fill (diastole) with blood before the next ejection is just as functionally important. The filling capacity of the heart is derived from two processes: active re-uptake of intracellular calcium that initiates the relaxation process and the ability of myocardium to stretch as one functional unit to accommodate the reception of blood from the atria. Impairments in contractility, relaxation, or compliance can lead to a decrease in systemic output of oxygenated blood and heart failure.

Essential to a diagnosis of heart failure is the presence of symptoms. After systolic and/or diastolic dysfunction manifests symptomatically and an echocardiographic assessment has been completed, a patient is grouped into a certain classification of heart failure based on ejection fraction. If calculated EF is <40% that patient is diagnosed with heart failure with reduced ejection fraction (HFrEF), whereas a patient with calculated EF ≥50% is diagnosed with heart failure with preserved ejection fraction (HFpEF) (9). Additionally, an EF between the two cut-off values (40-49%) places an individual in a heart failure with mid-range ejection fraction (HFmrHF); however, this is a novel category that is still under investigation (9). The larger divisions of heart failure diagnosis (HFrEF vs. HFpEF) both encompass a heart that is unable to meet systemic needs; however, the mechanism and etiology of the different diagnosis are mixed, with some features common to both, and others that predominate in one or the other.

Whereas an ischemic insult results in an intense local immune response that extends myocyte loss, ventricular chamber dilation, and systolic impairment (classic of HFrEF), the consequence of comorbidities as T2D, obesity, and a poor-quality diet that result in a low grade state of inflammation contribute to ventricular hypertrophy, stiffness, increased filling pressures, overt diastolic dysfunction and a restricted/hypertrophied ventricular cavity (more classic of HFpEF, Figure 2) (32,33). Despite the differing original insult to the myocardium, a common feature of both heart failure classifications is supranormal inflammation that alters cardiac structure and function in one form or another. Thus, greater understanding of the intricate biology of inflammation within the context of metabolic and cardiac dysfunction is essential.

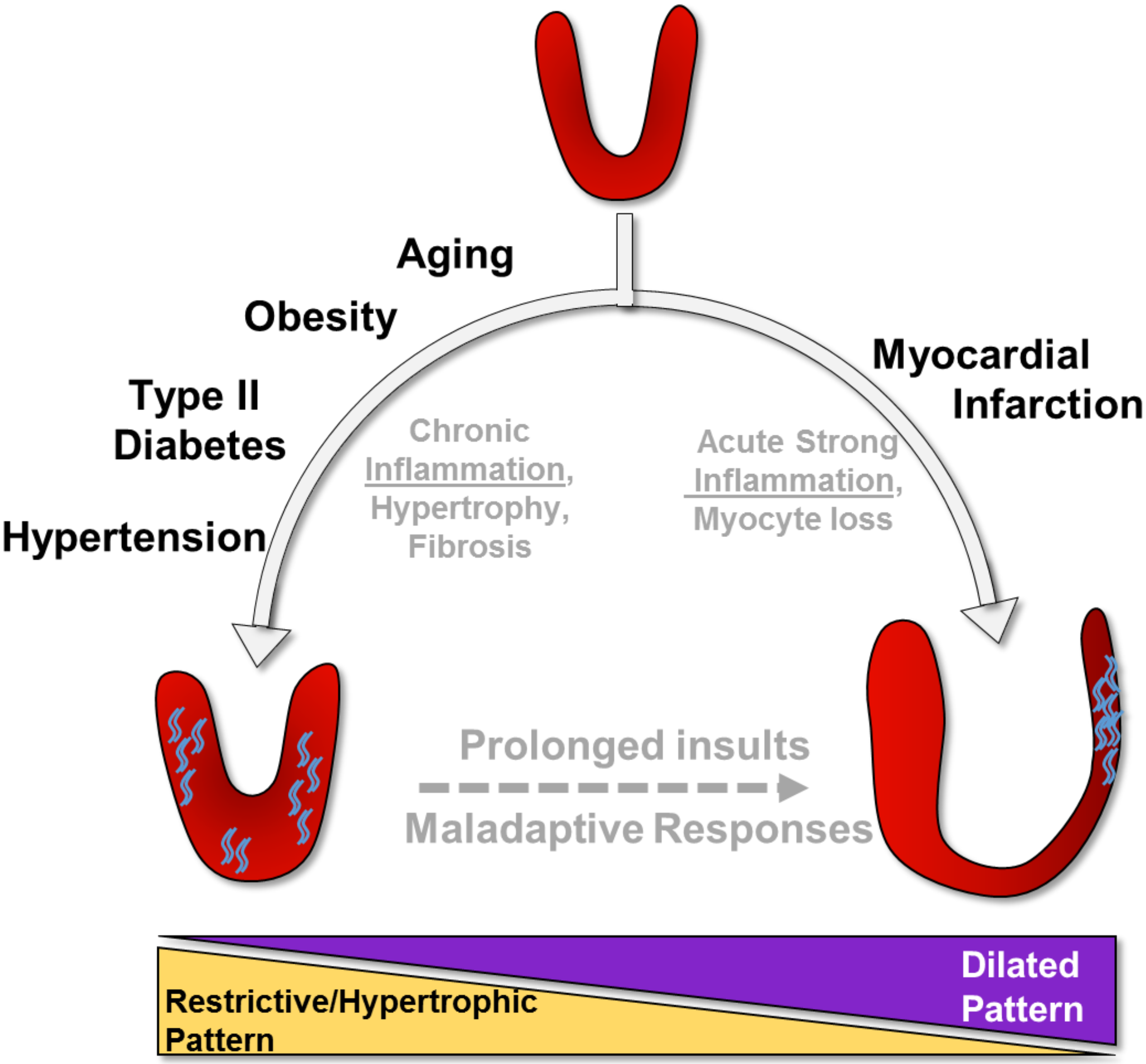
Aging
Obesity
Type II Diabetes
Hypertension
Chronic Inflammation, Hypertrophy, Fibrosis
Myocardial Infarction
Acute Strong Inflammation, Myocyte loss
Prolonged insults
Maladaptive Responses
Restrictive/Hypertrophic Pattern
Dilated Pattern

Figure 2. Differing cardiac insults lead to a spectrum of geometric and functional changes. Progression of cardiac structure and function based on differing initial insults. Acute ischemic damage (right) causes rapid and widespread myocyte cell death that sparks an intense local immune response. Secondary loss of nearby functional myocytes due to immune activation amplifies local damage. Ventricular volume increases in a Frank-Starling mechanism to increase preload to compensate for poor contractility, culminating in dilated LV and systolic dysfunction. Chronic systemic inflammation stemming from multifactorial combinations of aging, poor quality diet, obesity, and Type II Diabetes (T2D) (left) can cause myocyte hypertrophy and stiffness that restrict the ventricle’s ability to relax and reduce ventricular tissue compliance resulting diastolic dysfunction.

Epidemiologic studies to estimate new and total cases of a disease on a population scale serve to evaluate disease burden placed on the collective health care system and members. A recent population study evaluating the decade of 2000—2010 discovered a decline in incidence of overall heart failure diagnosis; however a categorical shift in incidences occurred. HFrEF reduced to a greater degree than HFpEF to the point that HFpEF is making up a larger proportion of new heart failure cases ([10], [11]). Problematic to this rise in HFpEF is the nearly matching high mortality rate at 5 years with HFrEF (65% vs. 68% respectively), and the disconnect that current therapeutic options for HFrEF patients do not appear to improve mortality in HFpEF patients ([12,13]). Perhaps due to the multifactorial nature of heart failure including aging, obesity, and hypertension that are exhibiting parallel prevalence increases, the overall prevalence of heart failure continues to rise in the United States ([11]).

Although established that obesity increases the *risk* of developing heart failure, *after the onset* of heart failure there in fact may be a *survival benefit* associated with being obese. Depicted in Figure 3, multiple studies have replicated this paradoxical finding of lowest mortality in heart failure patients to be patients that are obese (BMI 30-34.9 kg/m^2) ([14–18]). This apparent relationship is complex, but some varying hypothesis exist in attempts to explain this phenomenon including: a metabolic “reserve” that benefits obese patients during the catabolic state of heart failure, increased skeletal muscle mass, increased levels of adipocyte derived anti-inflammatory/insulin-sensitizing proteins ([19]), decreased sympathetic nervous system activation ([20]), or earlier identification of disease and better tolerance of protective medications ([21]). Interestingly, the paradox appears to be more apparent in patients with low cardiorespiratory fitness, with low CRF obese patients again having improved survival over low CRF normal BMI patients ([22]). Although unclear, the biological mechanisms that contribute to outcomes in patients with co-existing metabolic and cardiac dysfunction certainly warrant further investigation.

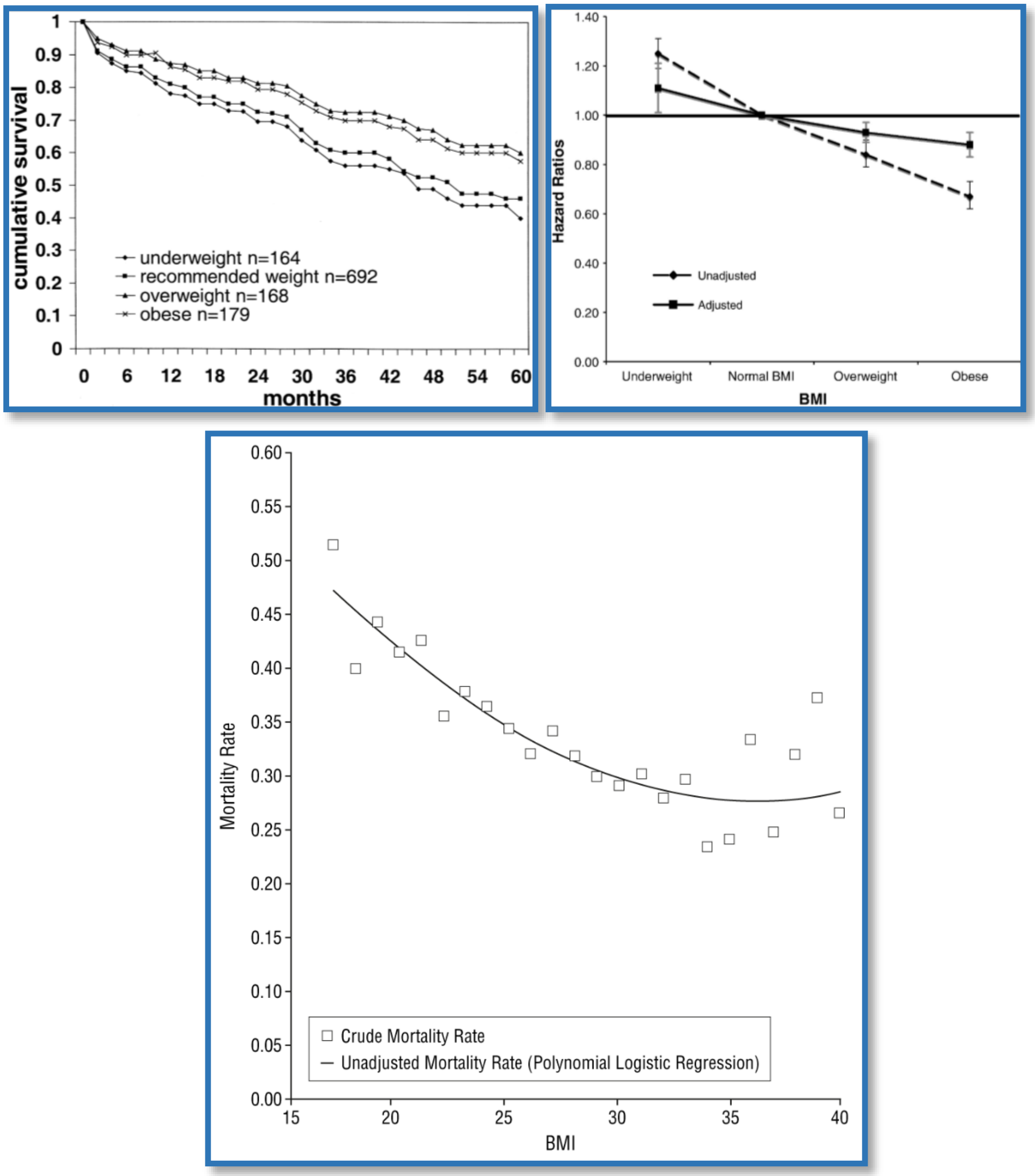

Figure 3. Mortality in Obese HF patients. Adapted from Horwich et al JACC 2001, Curtis et al JAMA 2005, and Oreopoulos et al AHJ 2008. Differing representations of improved survival in obese (BMI ≥ 30kg/m^2) patients that have diagnosed heart failure.

للاستشارات المنارة

Inflammation: obesity derived and heart failure mediator

The numerous adverse effects of obesity for development of morbid conditions including Type II Diabetes (T2D), coronary artery disease, heart failure, cancer, and more have been noted for decades. Elucidation of exact chronologic events that initiate a path toward obesity and end with a co-morbid condition have been elusive, but our understanding of changes in physiology during obesity have expanded.

The overlap of metabolic and immune system functions to homeostasis indicate that perturbations of one can have a maladaptive domino effect on the other. Individual observations in the clinic and within entire populations have depicted a clear connection between metabolic dysfunction and immune responses. Increases of BMI (overweight and obese) in men and women are positively associated with increased levels of high-sensitivity C-Reactive Protein (hs-CRP), a protein secreted by the liver in response to stress or injury that functions to augment acute innate immune activity ([23]). These observations have led to the catch-phrase describing obesity as a state of “low-grade chronic inflammation.”

When the balance of energy intake exceeds energy expenditure, due to any combination of increased whole caloric consumption, increased dietary energy density from higher saturated fat composition, or decreased physical activity, the capacity of adipose tissue to store the energy is exceeded. Due to the fact that adipose tissue is composed of a mix of adipocytes, networks of endothelial and smooth muscle cells, and resident macrophages and T-cells, changes in the integrity of adipocytes are rapidly sensed and transmitted systemically ([24]). Overwhelming the adipose storage depot leads to a detrimental cycle of adipocyte dysfunction and immune activation with whole body negative implications.

Depicted in Figure 4, multiple hypothesis exist for how adipocyte dysfunction manifests with subsequent immune activation and eventual metabolic dysfunction. Included are synergistic activity of adipocyte-derived cytokines, free fatty acid (FFA) release, endoplasmic reticulum (ER) and oxidative stress, and hypoxia from outgrowing local vascular supply (25). Prolonged adipocyte and immune cell shifts toward pro-inflammatory routes participate in local and systemic impairment of insulin sensitivity and glucose utilization, hallmarks of T2D and metabolic syndrome (26).

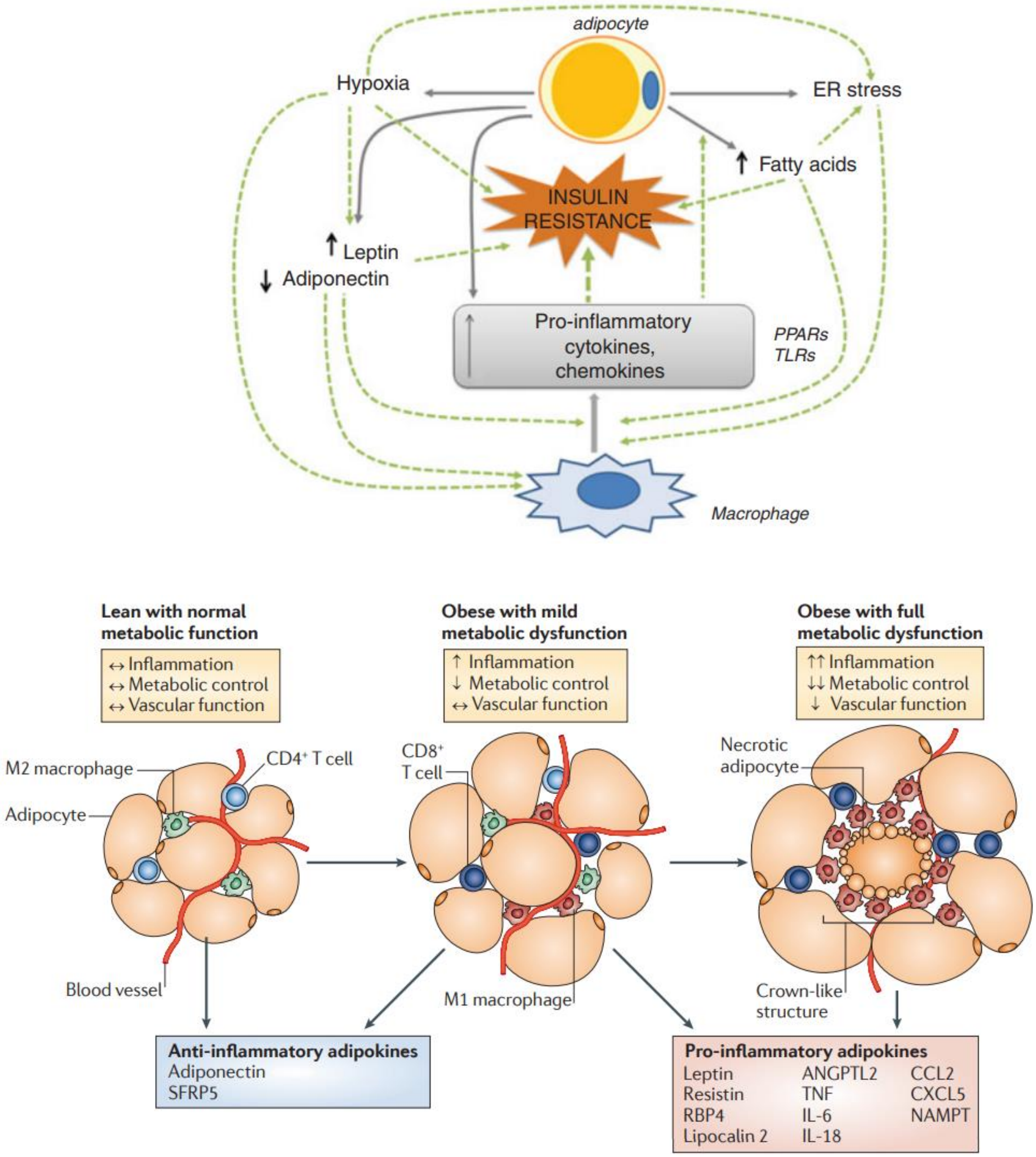
adipocyte
Hypoxia
ER stress
↑ Fatty acids
INSULIN RESISTANCE
↑ Leptin
↓ Adiponectin
Pro-inflammatory cytokines, chemokines
PPARs
TLRs
Macrophage
Lean with normal metabolic function
↔ Inflammation
↔ Metabolic control
↔ Vascular function
Obese with mild metabolic dysfunction
↑ Inflammation
↓ Metabolic control
↔ Vascular function
Obese with full metabolic dysfunction
↑↑ Inflammation
↓↓ Metabolic control
↓ Vascular function
M2 macrophage
CD4⁺ T cell
Adipocyte
Blood vessel
CD8⁺ T cell
M1 macrophage
Necrotic adipocyte
Crown-like structure
Anti-inflammatory adipokines
Adiponectin
SFRP5
Pro-inflammatory adipokines
Leptin
Resistin
RBP4
Lipocalin 2
ANGPTL2
TNF
IL-6
IL-18
CCL2
CXCL5
NAMPT

Figure 4. Inflammation from adipocytes. (Top) Adapted from Heredia et al Proceedings of the Nutrition Society 2012, Mechanisms for how adipocyte dysfunction communicates with local macrophages to perpetuate a state of inflammation that lead to insulin resistance. (Bottom) Adapted from Ouchi et al Nature Reviews: Immunology 2011, Development of obesity changes adipose tissue morphology and composition toward a milieu of pro-inflammatory cytokines and adipokines that derail homeostatic metabolism.

After growing evidence of increased cytokines such as TNF-α, IL-6, and other inflammatory components such as hs-CRP directly associated with increased heart failure incidence and worsening heart failure functional classification, it became apparent that the presence of inflammation in heart failure patients could be valuable targets to predict outcomes (27–29). Basic science observations of cytokines, such as TNF-α, IL-1β, and IL-18, causing ventricular remodeling, myocyte hypertrophy, decreasing contractility, and inducing apoptosis pushed the role of inflammation from association toward causative in the pathogenesis of heart failure (30–33). Discussed further is a major component of inflammation, Interleukin-18, and its contributions to immune, cardiovascular and metabolic function.

IL-18: Production and Signaling

Since first implicated in 1989 as a soluble factor that, in combination with IL-12, induced Interferon-γ in a mouse model of endotoxemia, our understandings of the signaling and function of Interleukin-18 in human *physiology* and *pathophysiology* have expanded (34). The Interleukin-1 family of cytokines, now encompassing 11 proteins of varying homology including IL-1β and IL-18, have been extensively studied for how they contribute to coordination of immune response. Unlike its IL-1 family member IL-1β, the human chromosome 11 and murine chromosome 9 gene product IL-18 has been identified to be constitutively expressed in human peripheral blood mononuclear cells (PBMCs, including T cells, B cells, natural killer (NK) cells and monocytes) as well as endothelial cells, keratinocytes, osteoblasts, dendritic cells, astrocytes, microglia and gut epithelial cells ([35],[36]). Depicted in Figure 5, The 24kD 193 amino acid product is synthesized without an endoplasmic reticulum signaling peptide, does not contain N-glycosylation sites, and remains inactive in the cytosol until cleavage by the cysteine protease Caspase-1 creates the 17kD 157 amino acid active form to be secreted out of the cell via plasma membrane pore or released by membrane rupture ([37–40]).

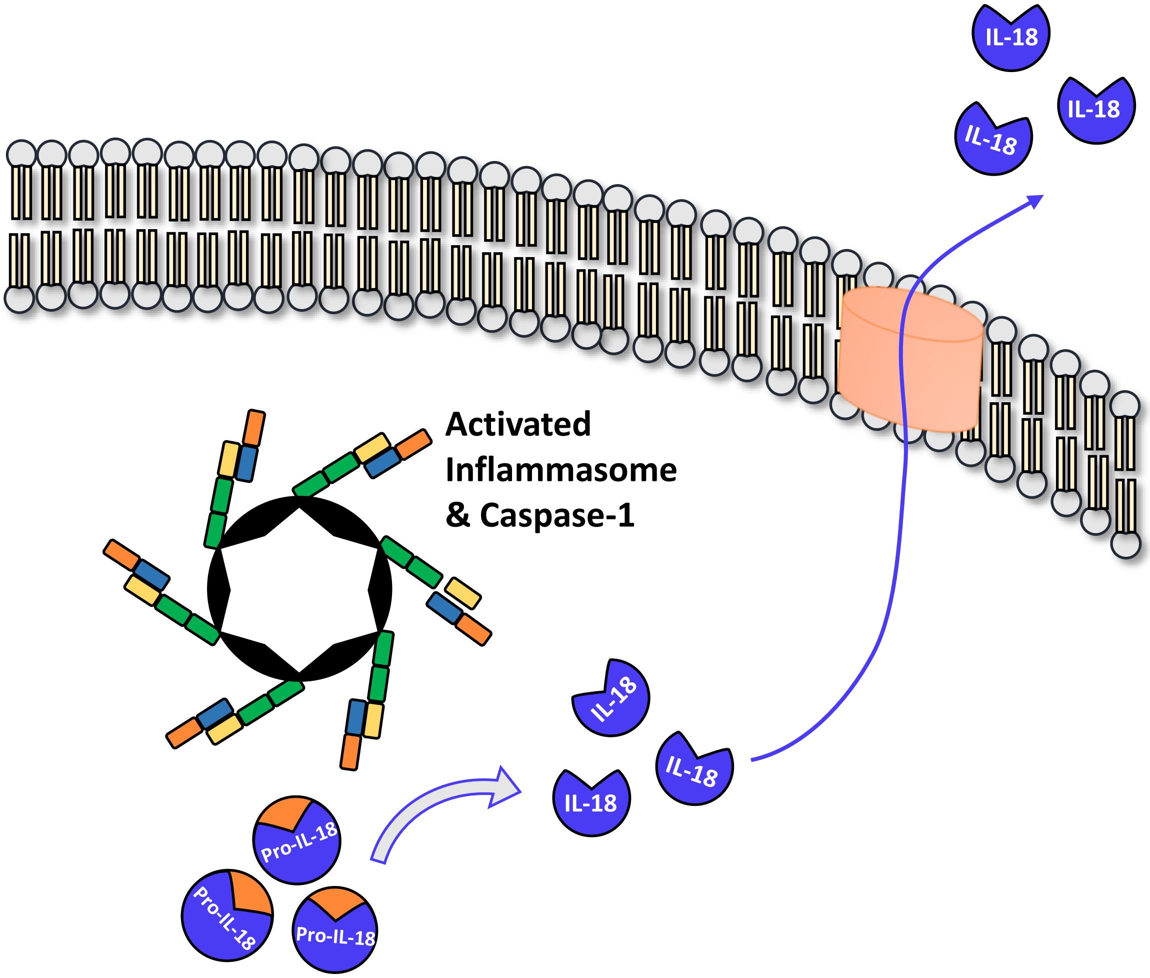

Figure 5. IL-18 Activation and Release. Activation of an Inflammasome yields an active Caspase-1 that is able to cleave Pro-IL-18 into its active form, IL-18. Secretion out of the cell is mediated by either release out of an intact cell membrane through a pore (shown here), or via cell membrane rupture, as during cell death.

While constitutive synthesis exists in the aforementioned cells, synthesis of IL-18 induced by stimuli is partially controlled by the transcription factor Nuclear Factor kappa-light-chain-enhancer of activated B cells, commonly NF-κB ([41]). Naturally then, a wide array of stimuli that converge upon the activation of NF-κB, including microbial and endogenous products binding to the Toll-like receptor (TLR) family, cytokines, and the antigen receptors T-cell Receptor (TCR) and B-cell Receptor (BCR), result in an increase in IL-18 production. Of note, the kinetics of IL-18 induction after TLR stimulation are different from IL-1β. Lipopolysaccharide (LPS) activation of TLR4 showed IL-18 levels reach maximal level at 8 hours and remain elevated at 24 hours, whereas IL-1β reaches maximum levels at 4 hours and decrease by 24 hours ([42]).

Once produced, activated, and secreted, IL-18 diffuses locally and is capable of acting in autocrine, paracrine, and endocrine fashions. With resemblance to IL-1β signaling, a heterodimeric receptor complex mediates conversion of the extracellular IL-18 signal to an intracellular response([43]). Depicted in Figure 6 below, the two proteins that complement each other to facilitate binding and signal transduction are IL-18Rα and IL-18Rβ. The alpha chain is expressed on a broad array of cell types, possess low affinity for IL-18, and alone is non-responsive to IL-18 *in vitro* ([43–45]). In contrast, the beta chain, known also as an accessory protein, is expressed in lung, spleen, prostate, small intestine, peripheral blood T cells and NK cells and has higher affinity for its IL-18 substrate ([45]). Alone, each receptor is unable to activate NF-κB or c-Jun N-terminal kinase 1 (JNK1, a regulatory protein for gene transcription); however, in combination the receptors elicit strong NF-κB and JNK1 activation ([45]). Additional intricacies of the IL-18 receptor complex, responsible for the research here, are discussed later.

Due to partial cytoplasmic homology of the IL-1 and IL-18 receptors, it is no surprise the two provoke a similar intracellular response ([43]). Intracellular cascades consist of myeloid differentiation primary response 88 (MyD88) recruitment to the receptor tail, IL-1 Receptor-Associated Kinase (IRAK) and TNF receptor associated factor 6 (TRAF6) interaction, and eventual NF-κB activation ([46–48]). Distinct again from IL-1 signaling is the additional activation of mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3) with IL-18 stimulus as evidenced by *in vitro* studies of NK cell lines ([49]).

Also integral to net IL-18 signaling are the soluble circulating endogenous protein isoforms, known as IL-18 Binding Protein (IL-18BP), that bind free extracellular IL-18 in a 1:1 ratio and remove it from the pool of available and functional IL-18 ([50]). Constitutively secreted at levels approximately 20 times that of IL-18 and with high affinity for IL-18, IL-18BP functions seemingly as a natural balance to the activation of the T helper cell response to prevent a detrimental autoimmune cycle ([51–53]).

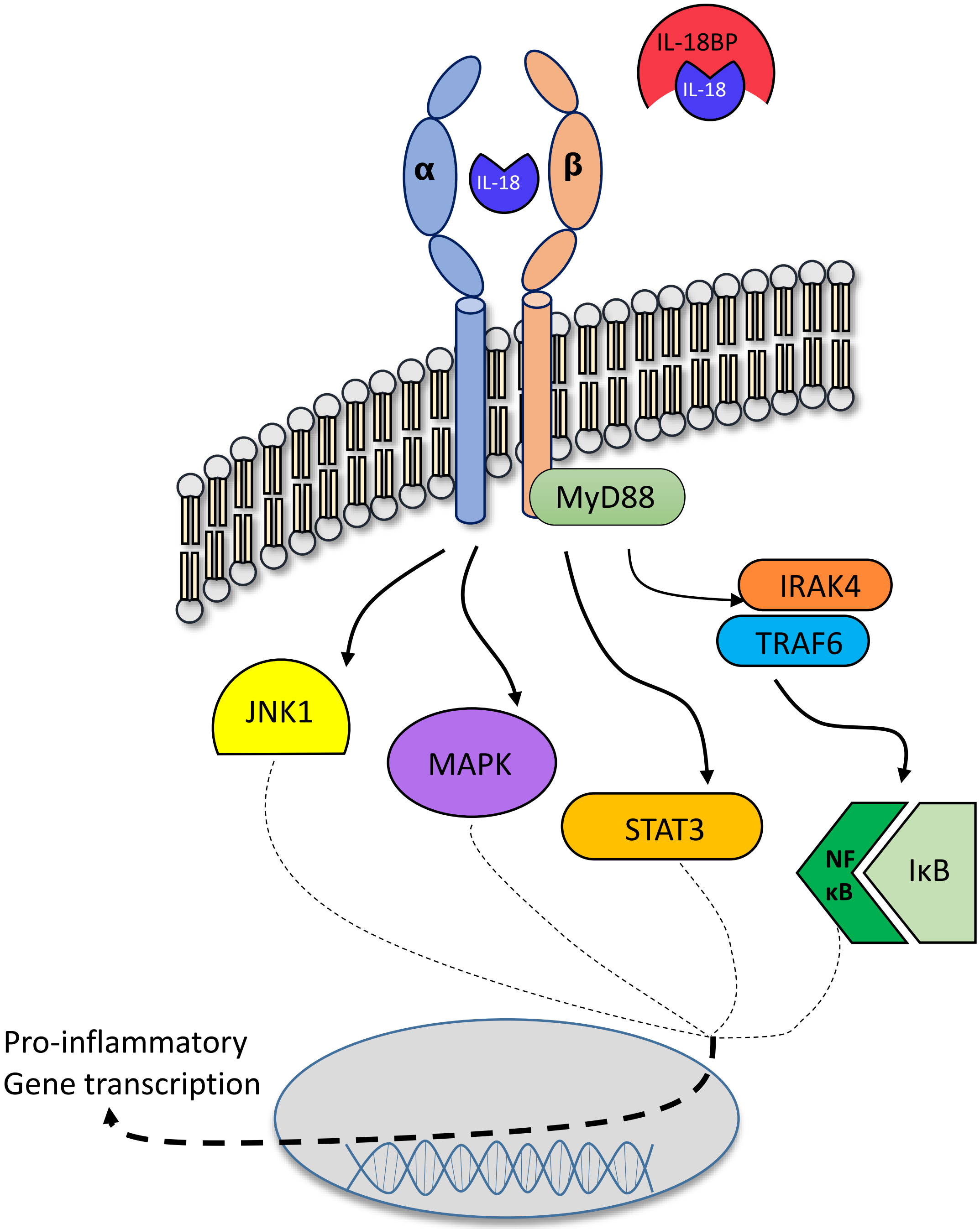
IL-18BP
IL-18
α
IL-18
β
MyD88
IRAK4
TRAF6
JNK1
MAPK
STAT3
NF κB
IκB
Pro-inflammatory
Gene transcription

Figure 6. IL-18 Binding and Signaling. Once in the extracellular space, free and active IL-18 (not bound to circulating IL-18BP) binds to its receptor complex composed of, at least, IL-18Rα and IL-18Rβ. Activation of the receptor complex leads to intracellular cascades including the activation of MyD88, IRAK4, TRAF6, JNK, MAPK, STAT3, and NF-κB, which facilitate transcription of genes involved in numerous cell functions, including inflammation.

IL-18: Functions

Role of IL-18 in the Immune System

The uniqueness and complexity of IL-18 biology is derived from its pleotropic effects on multiple cells and organ systems as well as the intricate balance between homeostatic and pathologic functions. In regards to the immune system, IL-18 has its hand in innate, adaptive, and autoimmunity; responsible for activation and/or differentiation of multiple cell types including macrophages, naive T cells, NK cells, and B cells ([54]). Specific cytokine environments coordinate and specialize immune cell responses and contribute to acute disease states (such as myocardial infarction, microbial invasion of the gut, and skin lesions), chronic disease states (such as heart failure and Type II diabetes mellitus) and genetic mutations (macrophage activation syndrome) ([54–56]).

Inflammation is an evolutionarily refined process initiated by the immune system designed to clear local and global pathogen or damage insults and best attempt to restore tissue to its previous functional state. The ability of IL-18 to induce chemokine production to attract neutrophils ([57]), increase endothelial Intercellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), and E-Selection expression ([58, 59]), and stimulate production of IL-6 and IL-1β place it central to initiation and propagation of inflammation in various disease states ([60]). Innate immune cell products, such as IL-18, serve also to bridge the innate and adaptive immune responses. Pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide or bacterial DNA, or damage associated molecular patterns (DAMPs), such as extracellular ATP or mitochondrial DNA, bind to a family of pattern recognition receptors (PRRs) that include the Toll-Like Receptors (TLRs). Binding of ligands to TLRs present on neutrophils and macrophages initiates intracellular cascades leading to production of cytokines, including IL-18. Interleukin-12 importantly acts to increase plasma membrane IL-18Rα levels in CD4+ T cells, rendering the naïve T-cells able to bind extracellular IL-18. Binding of Il-18 to the IL-18 receptor complex and subsequent intracellular cascades culminate in transcription, translation, and release of INF-γ from the T-cell ([61]). IL-18 induced release of INF-γ further results in activation of macrophages to begin a pro-inflammatory feedback mechanism to amplify IL-18, IL-1β, and TNF-α levels, the defining characteristics of the Th1 response ([62]).

Role of IL-18 in the cardiovascular system: Health and Disease

Little evidence exists for the function of IL-18 in homeostasis and preservation of the myocardium or vasculature. The identification of pro-inflammatory cytokines, including IL-18, as factors associated with diseases such as hypertension, atherosclerosis, myocardial infarction, and heart failure has been studied extensively in the clinical and pre-clinical realms.

Hypertension

Hypertension is now present in approximately 46% of U.S. adults with varying etiologies (63). In response to prolonged hypertension, the myocardium undergoes physiologic hypertrophy to decrease wall stress and oxygen consumption in order to continue ejecting adequate volumes of blood without entering into an ischemic state (64). However, sustained pressure overload tips the physiologic hypertrophic adaptation to pathologic as further hypertrophy stiffens the ventricle, impairs coronary circulation, and can lead to arrhythmias. Evidence of this hypertrophy discovered on electrocardiographic or echocardiographic assessment is an independent risk factor for future cardiovascular morbidity and all-cause mortality (65–67).

Clinical correlations and basic science investigation have identified IL-18 to be involved in the hypertrophic response (Figure 7, below). In patients, increased circulating IL-18 is correlated with hypertension as well as left ventricle mass (68). In a mouse model of pressure overload induced by thoracic aorta constriction (TAC), the genetic deletion of IL-18 (IL-18KO) resulted in less hypertrophy than wild-type control; however, was accompanied by worse LV contractility, indicating that in response to pressure overload

IL-18 is necessary to some degree for hypertrophic adaptations (69). A molecular pathway identified in hypertrophy induced by IL-18 is the PI3K→Akt→GATA4 signal that leads to transcription of genes including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (MHC), presumably to combat pressure overload (70, 71). *In vitro*, cyclical mechanical stress of cardiomyocytes increases mature IL-18 that is blunted with neutralizing antibodies or IL-18BP while *in vivo*, pressure overload increased myocardial IL-18 mRNA and protein, but decreased IL-18BP mRNA and protein (71). To further support the notion of IL-18 directly involved in the hypertrophic response, daily administration of IL-18 to young healthy mice induced left ventricular wall thickness and diastolic dysfunction (72,73). The expanding understanding of IL-18 in mediating hypertensive induced cardiac remodeling places further investigation of IL-18 signaling at the forefront of cardiovascular research.

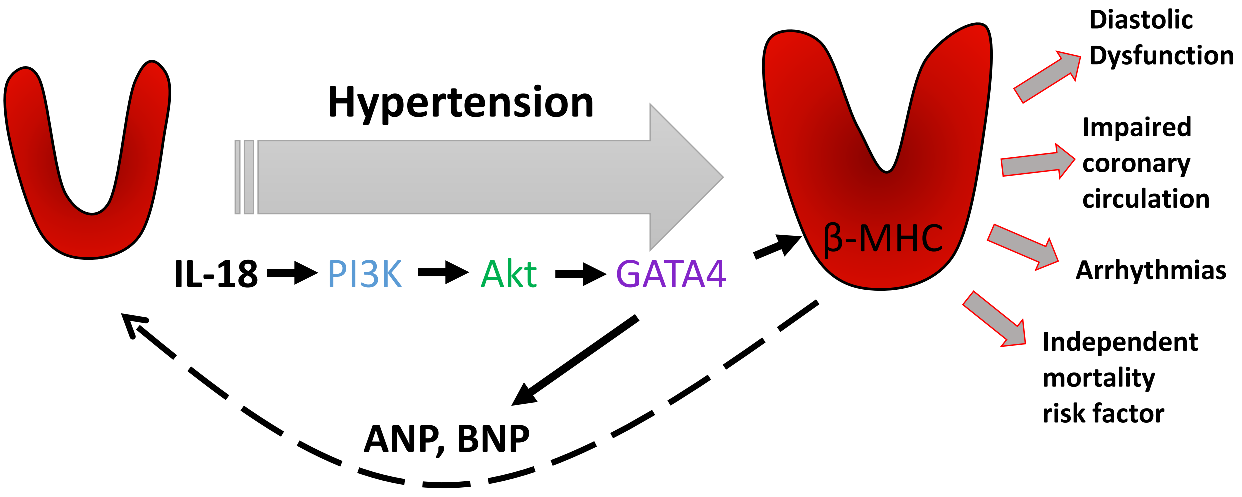
Hypertension
IL-18
PI3K
Akt
GATA4
β-MHC
ANP, BNP
Diastolic Dysfunction
Impaired coronary circulation
Arrhythmias
Independent mortality risk factor

Figure 7. Hypertrophic Adaptation and IL-18. IL-18 is capable of signaling cascades leading to physiologic hypertrophy and diuresis to adapt to elevated afterload. Sustained hypertrophy; however, impairs ventricular function and increases mortality risk.

Atherosclerosis

Atherosclerosis is increasingly defined as an inflammatory disease. Disease progression is characterized by damaged endothelium, due to shear stress (hypertension), oxidative stress from increased lipid metabolism (hyperlipidemia) or cholesterol accumulation (hypercholesterolemia), that attracts neutrophils to hone and activate macrophages and T lymphocytes that amplify local inflammation resulting in advanced complicated lesions in vessel walls ([74,75]). IL-18 has an integral role in gradual progression of lesions as well as the latter stages of plaque instability and rupture (Figure 8, below). Endothelium that has accumulated low-density lipoproteins and its derivatives are prone to oxidative stress and release of cytokines. IL-18 induced increases in adhesion molecules (ICAM-1) on the plasma membrane of the endothelium promote monocyte and T-cell adhesion and extravasation into the vascular intima where lipids are accumulating.

The aforementioned combination of local IL-12 and IL-18 drive the differentiation of naïve T-cells into the Th-1 phenotype to produce INF-γ within the vascular intima. Macrophage activation via INF-γ polarizes that cell line to a pro-inflammatory state, deemed the M1 response. Macrophages become significant local effector cells contributing to smooth muscle cell proliferation, necrosis of the plaque core, and extra-cellular matrix breakdown ([76]). Unstable/symptomatic plaques have shown increased levels of IL-18mRNA compared to stable/asymptomatic plaques in patients ([77]). Inhibition of IL-18 with an IL-18BP expression plasmid decreased lipid deposition, macrophage and T-lymphocyte infiltration, and increased collagen content in mice, further indicating that IL-18 is involved in plaque development and stability ([77,78]).

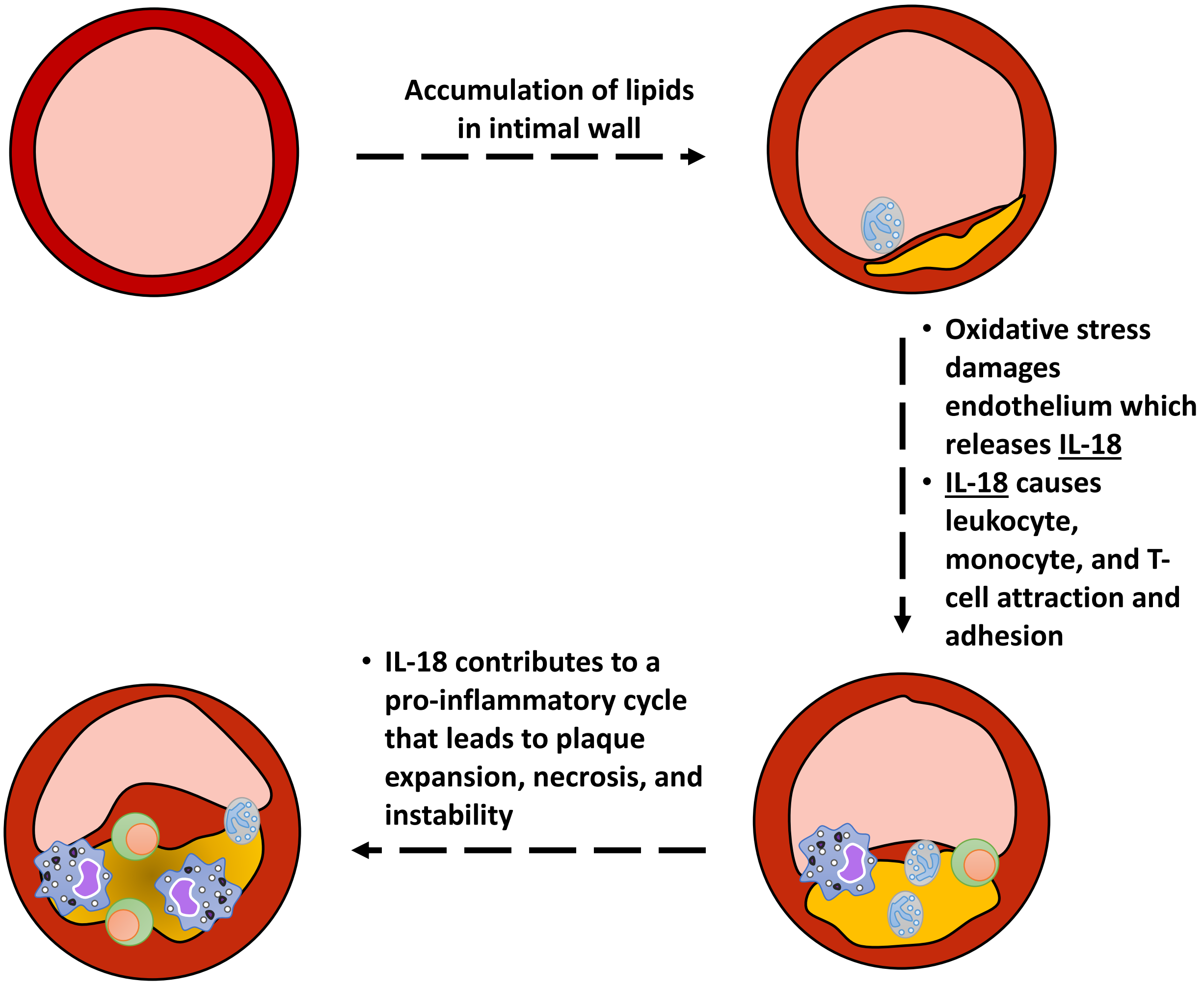
Accumulation of lipids in intimal wall
• Oxidative stress damages endothelium which releases IL-18
• IL-18 causes leukocyte, monocyte, and T-cell attraction and adhesion
• IL-18 contributes to a pro-inflammatory cycle that leads to plaque expansion, necrosis, and instability

Figure 8. Atherosclerosis and IL-18. Initial LDL and lipid accumulation within the vascular intimal wall occurs over a prolonged period. Extended exposure of endothelial cells to lipids results in oxidative stress, endothelial damage, and initiation of pro-inflammatory cascades, including IL-18, which recruits immune cells to the damage site. Local inflammation is amplified and leads to cell death within the plaque core and proliferation of surrounding smooth muscle cells that further narrow the vessel. Exacerbation past this point can create symptomatic ischemia with exercise or at rest.

Myocardial Infarction

After plaque rupture, coronary vessel occlusion, and myocardial ischemia, the crosstalk between immune cells and surviving myocytes and fibroblasts is essential to best repair the cardiac tissue. While essential to a degree, prolonged and exaggeration of this response is detrimental as it continues to destruction of remaining cardiomyocytes, expands infarct size, and accelerates the progression to heart failure ([79]). If the immune system response teeters the myocardium on the delicate balance between repair and worsening prognosis, it comes logically that suppression of an exaggerated immune response could be beneficial ([80]). While IL-1β has received more investigation and therapeutic attention in this setting, IL-18 is also a key player.

After ischemia reperfusion in the mouse, cardiac, endothelial, and smooth muscle cell IL-18 mRNA is increased, as well as serum IL-18 levels ([81,82]). Pre-treatment with an IL-18 neutralizing antibody or injection of mesenchymal stem cells overexpressing IL-18BP into the coronary artery of mice reduced infarct size and improved ejection fraction ([82,83]). The cardiodepressant effects of IL-1β have been shown to be mediated by induction of IL-18, as IL-18KO mice treated with IL-1β did not have reduced fractional shortening compared to wild-type mice treated with IL-1β ([32]). Therefore, where treatment with IL-1R blockade improves cardiovascular outcomes in patients with previous myocardial infarction (MI), and IL-1 effects are in part mediated by IL-18, it is possible that inhibition of IL-18, either with exogenous binding protein or neutralizing antibody, could also be a treatment that yields meaningful clinical benefits to patients who achieved reperfusion after MI ([84]).

Heart Failure

Regardless of etiology, a staple of a failing heart is damaged or stressed myocardium that recruits and activates effector immune cells ([79]). Cytokine signaling between immune, fibroblast, endothelial, and cardiac cells induces altered calcium signal coordination, oxygen free radical damage, apoptosis, and myofibroblast expansion that combine to impair systolic and/or diastolic function and manifest as clinically relevant symptoms ([85]).

Clinical and basic science observations have identified elevated IL-18 to be associated with worsening heart failure functional class and involved in the pro-fibrotic response that contributes to diastolic dysfunction ([86, 87]**)**. Carbone et. al. have shown that IL-18KO mice on a high-saturated fat diet have preserved diastolic function despite greater obesity ([86]). In a rat model of metabolic syndrome and IL-18 overexpression, worsened diastolic dysfunction but preserved ejection fraction was noted ([88]).

A classic characteristic of heart failure is over activation of the sympathetic nervous system (SNS) that attempts to compensate for a failing heart's inability to perfuse tissue as it once could. The interplay between sympathetic activation and ensuing immune activation that perpetuates a failing heart, specifically involving IL-18, has been investigated in humans and mice (Figure 9, below). After noting an association between increased serum norepinephrine and IL-18 in patients with non-ST-segment elevation myocardial infarction (NSTEMI) or unstable angina, Xiao et. al. observed that acute over-activation of β-AR receptors with isoproterenol in mice induced myocardial IL-18 and macrophage infiltration ([89]). Blockade of IL-18 using antibodies reduced chemokine, adhesion molecule, and pro-inflammatory cytokine gene expression, as well as reduced cardiac inflammation, fibrosis, and improved functional parameters ([89]).

Additionally involved in the circulatory system's response to heart failure is activation of the renin-angiotensin-aldosterone system (RAAS), again in attempt to maintain cardiac output by retaining Na^+ and arterial vasoconstriction. There is increasing evidence of hormonal (Angiotensin II) and inflammatory (IL-18) convergence onto pathways causative of diastolic dysfunction. Angiotensin II increases myocardial fibrosis in mice, partially through production of Osteopontin (OPN). OPN is a protein secreted by macrophages, fibroblasts, and myocytes into the extracellular space that is critical to collagen synthesis and deposition, as well as myofibroblast differentiation ([90]). IL-18 administration to mice has also been shown to increase OPN levels, along with myocardial fibrosis, collagen, and ventricular stiffness (Figure 9, below) ([91]).

In summary, basic science models and patient cohorts have represented IL-18 involvement in development and worsening of cardiovascular diseases including hypertension, atherosclerosis, myocardial infarction, and heart failure. Further clarification of IL-18 pathways may pay great dividends going forward for patients at risk for and those who have already developed the aforementioned diseases.

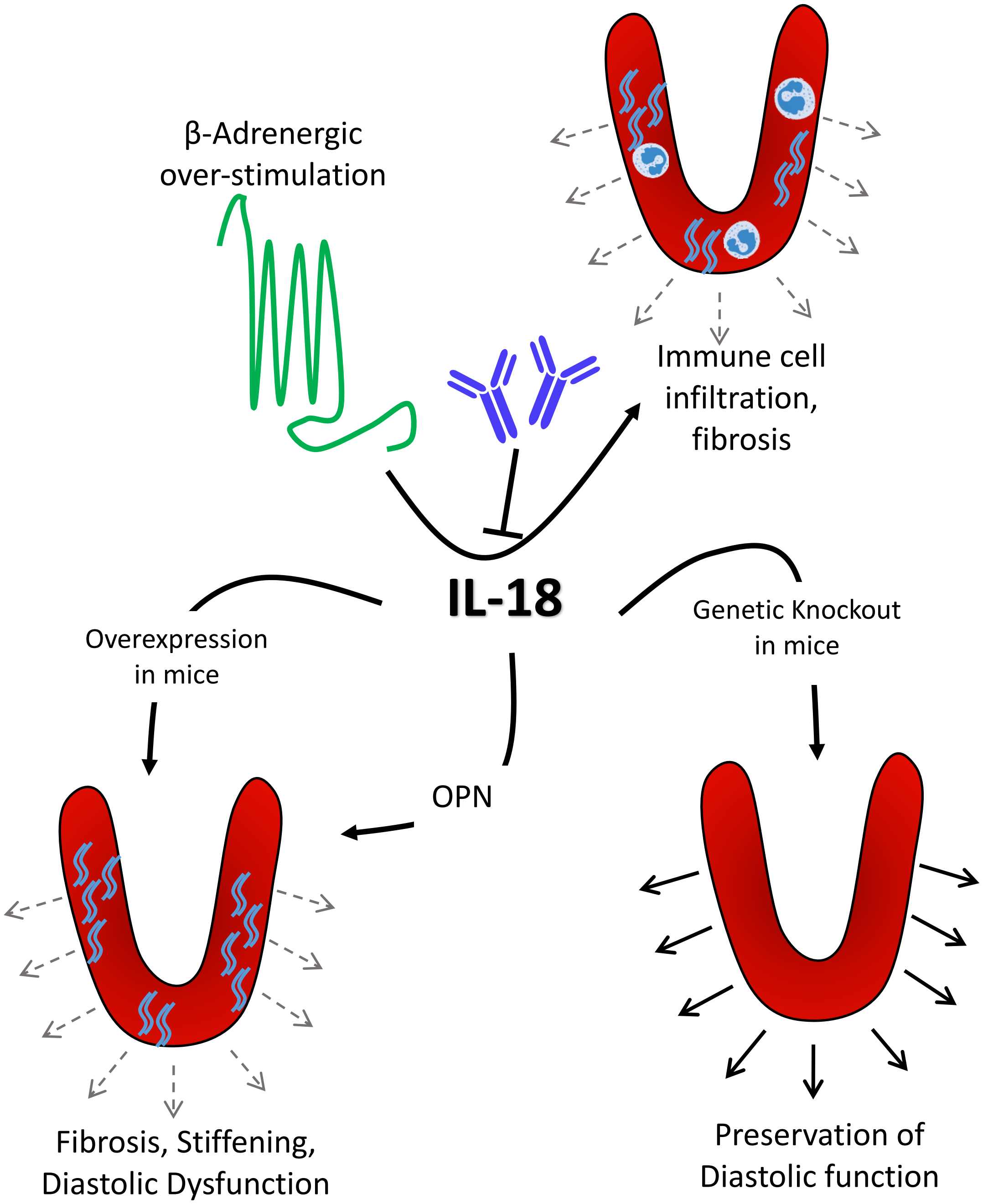
β-Adrenergic over-stimulation
Immune cell infiltration, fibrosis
IL-18
Overexpression in mice
Genetic Knockout in mice
OPN
Fibrosis, Stiffening, Diastolic Dysfunction
Preservation of Diastolic function

Figure 9. IL-18 and diastolic dysfunction. Overexpression of IL-18 and induction of the protein Osteopontin (OPN) leads to fibrosis and diastolic, while genetic knockout of IL-18 in a model of diet-induced obesity preserved diastolic function and reduced fibrosis ([80]). Stimulation of β-Adrenergic receptors with isoproterenol induces immune cell recruitment and diastolic dysfunction that was preserved with antibody blockade of Il-18 ([82]).

Role of IL-18 in metabolism: Health and Disease

The complicated nature of IL-18 signaling in metabolism is in part due to contrasting physiological observations. In the early 2000's, clinical correlations between circulating IL-18 levels and obese and diabetic patients were made. Compared to healthy volunteers, patients with type 2 diabetes mellitus had higher serum IL-18 (92). Obese women were also found to have higher serum IL-18 levels that positively correlated with BMI, waist-to-hip ratios, and negatively correlated with insulin sensitivity (93). Acute hyperglycemia in healthy and glucose intolerant patients showed an increase in IL-18 levels that was prolonged in patients with impaired glucose tolerance (94). A separate study incorporating a large community population indicated a consistent graded relationship between plasma IL-18 and odds ratio for metabolic syndrome (95).

This discovery of IL-18 *positively* correlating with the metabolic syndrome in patients has led to a growing number of preclinical investigations. Early evaluation by Netea et. al. in IL-18KO mice showed the spontaneous development of obesity and insulin resistance at 6 months of age that was preceded by increased chow intake (hyperphagia) (96). Supporting data by Zorrilla et. al. again showed that IL-18KO mice developed a phenotype of increased body weight and adiposity preceded by increased standard chow or high fat diet intake, as well as an increased respiratory exchange ratio (RER) (97,98). Collectively, these initial results indicated that although clinical data suggested a *pathophysiologic association* of IL-18 in the development of the metabolic syndrome, the genetic *absence* of IL-18 was also able to produce a phenotype of obesity and insulin resistance.

Recent work has further implied IL-18 to be essential to body weight and glucose homeostasis through catalytic activation by the NLRP1 inflammasome in the mouse. Collectively, Murphy et.al. was able to show NLRP1's ability to respond to energy intake and coordinate metabolic homeostasis, specifically through IL-18 (99).

Interestingly, administration of recombinant IL-18 (rIL-18) to mice via intravenous administration had no effect on food intake while intracerebral administration significantly reduced food intake, suggesting the effect of IL-18 on food intake may occur within the central nervous system (CNS) (96,98). After elegant work discovered precise neurocircuit elements responsible for feeding behavior in the lateral hypothalamus (LH), the central effects of IL-18 on appetite were further investigated (100,101). It was discovered that IL-18 altered neuron activity in the (LH) to result in decreased food intake in mice (101).

Peripherally, IL-18 administration to mice has been shown to slightly increase insulin sensitivity, increase 5' AMP-activated protein kinase (AMPK), and increase fatty acid oxidation (96,102). Additionally, rIL-18 administration to mice decreased body weight and improved lipid handling capacity of the liver (103). Thus, an overall divide of IL-18 metabolic contributions between the CNS to regulate food intake and the periphery to positively alter insulin sensitivity and lipid metabolism has been discovered (Figure 12).

The apparent *negative* contributions of IL-18 to cardiovascular disease mixed with possible *positive* influences on metabolism, namely decreased food intake and improved glucose/lipid metabolism, create an unclear therapeutic potential of IL-18. On one hand, inhibition of IL-18 signaling at the myocardium appears beneficial, as it has been shown to attenuate cardiac dysfunction. However, genetic absence of IL-18 in mice leads to overt obesity and T2D. Thus, an apparent disconnect in IL-18 signaling exists (Figure 12).

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Similarities and differences in IL-18KO and IL-18RαKO mouse phenotypes: A role for the Na-Cl Co-transporter?

As discussed before, deletion of the ligand IL-18 (IL-18KO) results in a phenotype of obesity and T2D (96,98). However, mildly differing phenotype results have been observed after the genetic deletion of the IL-18Rα, responsible for binding IL-18. IL-18RαKO mice display hyperinsulinemia and decreased glucose tolerance akin to IL-18KO mice; however, regardless of diet, they exhibit body weight changes and food intake levels that resemble wild-type mice more than IL-18KO mice (Figure 10) (102, 104).

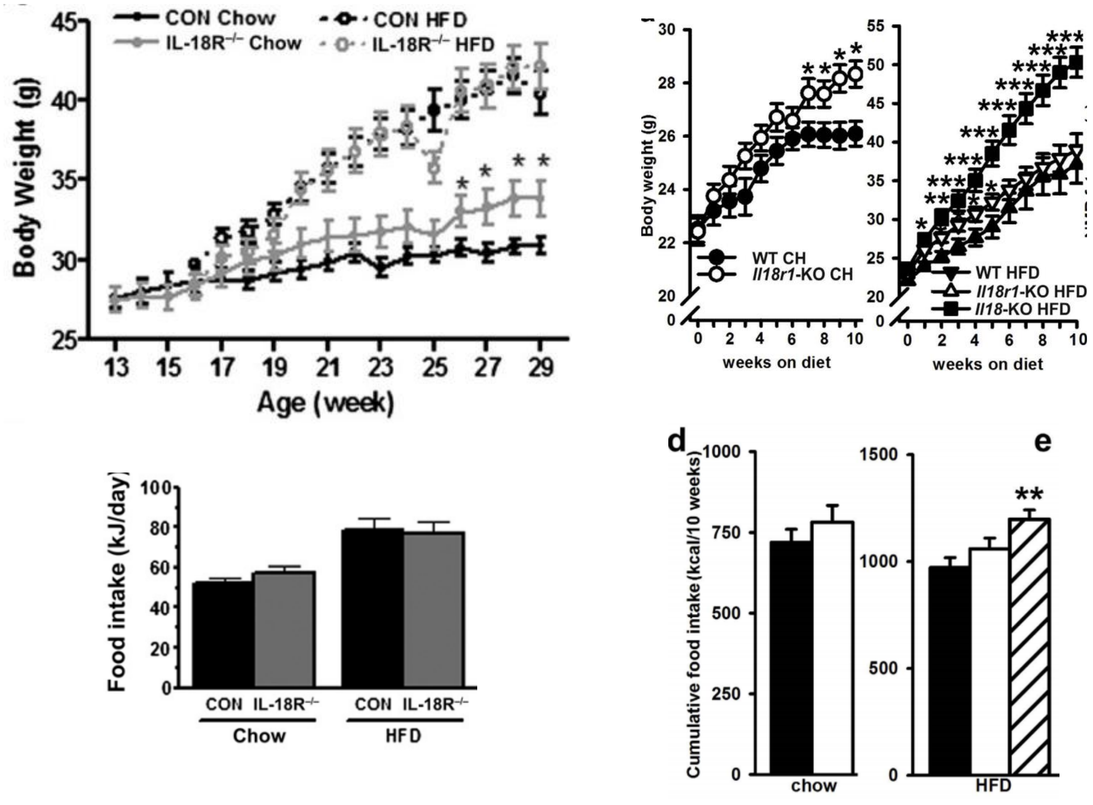

Figure 10. IL-18KO and IL-18RKO divergence. Adapted from Lindegaard et al Diabetes 2013 and Pazos et al Scientific Reports 2015. (Top) Body weight gain on either standard diet (SD, CH, chow) or high-fat diet (HFD) of wild-type, IL-18RαKO, or IL-18KO mice. Apparent is the trend that IL-18RαKO mice do not have a growth pattern that is alike IL-18KO mice. (Bottom) Chow or HFD food intake of wild-type, IL-18RαKO, or IL-18KO mice. Again, the IL-18RαKO mice do not exhibit hyerphagia like IL-18KO mice.

Additionally, peripheral disconnects in IL-18 signaling have been shown in the context of atherosclerosis. In a mouse model of atherosclerosis, IL-18RαKO was unable to decrease plaque size *in vivo* or prevent IL-18 binding to isolated endothelial cells *ex vivo*. A cell surface binding alternative for IL-18 was found to be the 125 kDa Na-Cl Co-transporter (NCC) (105). The distal convoluted tubule (DCT) apical transmembrane protein is known to be responsible for ~5% of Na+ reabsorption in the kidney and is the target for thiazide-type diuretics that are considered to be part of first-line therapy for hypertensive patients (106,107).

Further investigation yielded that IL-18 had strong binding affinity for NCC, and IL-18 treatment *in vitro* to cells expressing NCC and not IL-18Rα increased protein tyrosine phosphorylation, indicating a signal is transduces across the plasma membrane due to IL-18 binding to NCC. Genetic changes in the mouse model of atherosclerosis yielded further support to *in vitro* IL-18/NCC communication observations. Decreases in atherosclerotic lesion are were only observed in the double knockout of *Apoe*$^{-/-}$*NCC*$^{-/-}$*IL-18Rα*$^{-/-}$ and not in *Apoe*$^{-/-}$*NCC*$^{-/-}$ or *Apoe*$^{-/-}$*IL-18Rα*$^{-/-}$ backgrounds (Figure 11, below). Combined, these results indicated that the membrane localized Na-Cl Co-transporter effectively played a role in mediating atherosclerosis through IL-18 signaling in the mouse, suggesting that NCC may also mediate other physiologic processes where IL-18 signaling is important, possibly including metabolic and cardiac function discussed previously.

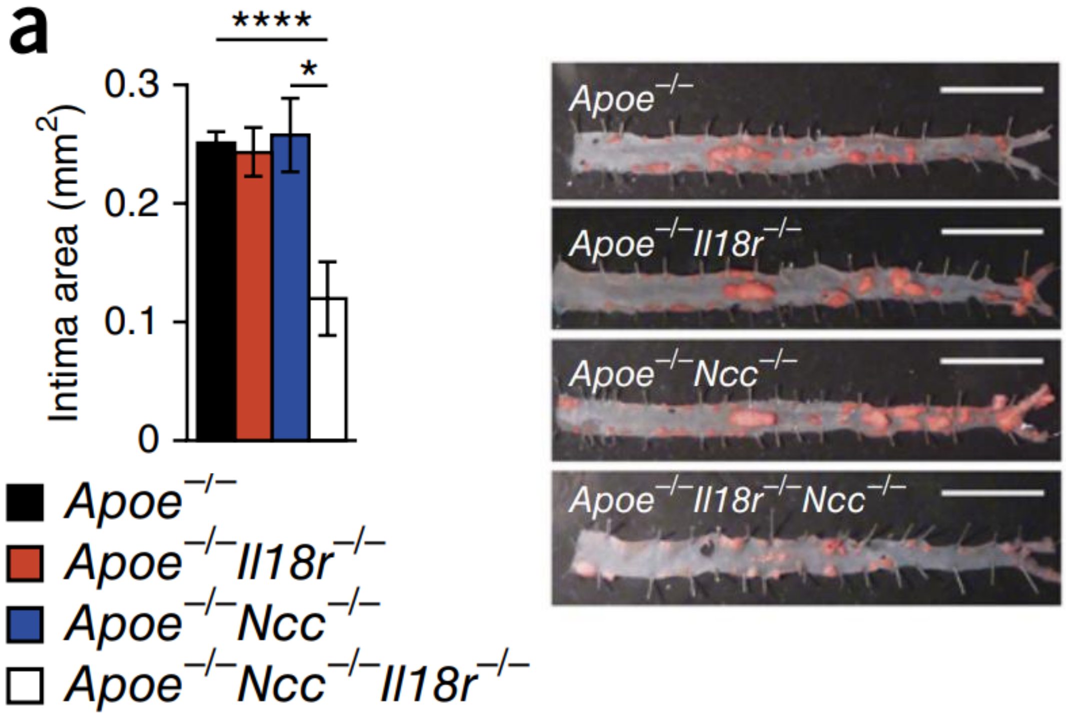

Figure 11. NCC in Atherosclerosis. Adapted from Wang et al Nature Medicine 2015, Aortic root lesion intima area in different genetic backgrounds. Where Apoe−/−NCC−/− or Apoe−/−IL-18Rα−/− backgrounds are not protected from atherosclerosis, Apoe−/−NCC−/−IL-18Rα−/− significantly decreases aortic lesion area intimal area. These results implicate NCC as a receptor in physiologic processes where IL-18 activity is involved.

Thus, further understanding the mechanism by which IL-18 signaling is achieved may reveal intricacies to its biology, role in homeostasis, and metabolic and cardiac disease as well as open therapeutic doors (Figure 12, below). The major goal of the work presented here, and still underway, is aimed at unraveling the role of NCC as a co-receptor for IL-18 signaling in metabolic and cardiac function by investigating the genetic knockout of NCC in a mouse model of high-fat diet induced obesity.

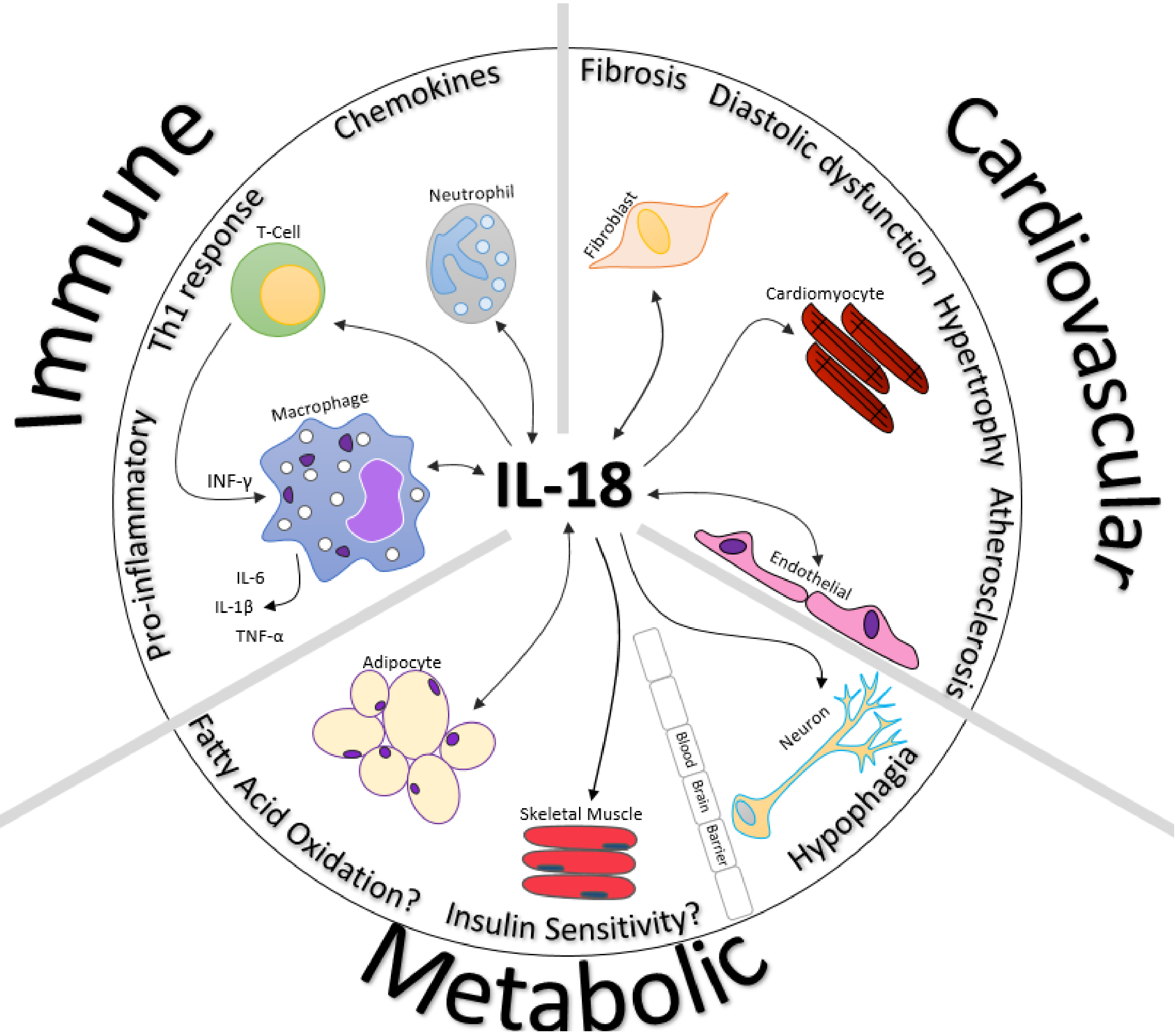
Immune
Chemokines
Fibrosis
Diastolic dysfunction
Cardiovascular
Th1 response
T-Cell
Neutrophil
Fibroblast
Cardiomyocyte
Hypertrophy
Pro-inflammatory
Macrophage
INF-γ
IL-18
IL-6
IL-1β
TNF-α
Endothelial
Atherosclerosis
Adipocyte
Neuron
Blood
Brain
Barrier
Hypophagia
Skeletal Muscle
Fatty Acid Oxidation?
Insulin Sensitivity?
Metabolic

Figure 12. IL-18 Physiology. The complex interplay of IL-18 and its contributions to immune, cardiovascular, and metabolic function. Within the immune system, IL-18 mediates communication between cell types to specialize the immune response toward a pro-inflammatory state. In the vascular system, IL-18 functions between endothelial, macrophages, and T-cells to promote atherosclerosis. Within the heart, IL-18 can induce cardiomyocyte hypertrophy and fibroblast collagen deposition that contribute to diastolic dysfunction. The effects of IL-18 on metabolism are less clear-cut, but include decrease food intake within central nervous system and possible positive modulation of glucose and lipid metabolism in the periphery at adipose and skeletal muscle tissue. The role of NCC as a receptor that mediates these functions is investigated here.

With the comprehensive background of Interleukin-18 being required for metabolic homeostasis, but able to cause ventricular hypertrophy, fibrosis, and dysfunction with the capability to act through its co-receptor Na-Cl Co-transporter (NCC) to elicit function; we hypothesize NCC may act to mediate IL-18 effects on metabolic and cardiac function. Therefore, the genetic knockout of NCC (NCCKO) will worsen the metabolic phenotype, but attenuate the cardiac phenotype after western diet feeding.

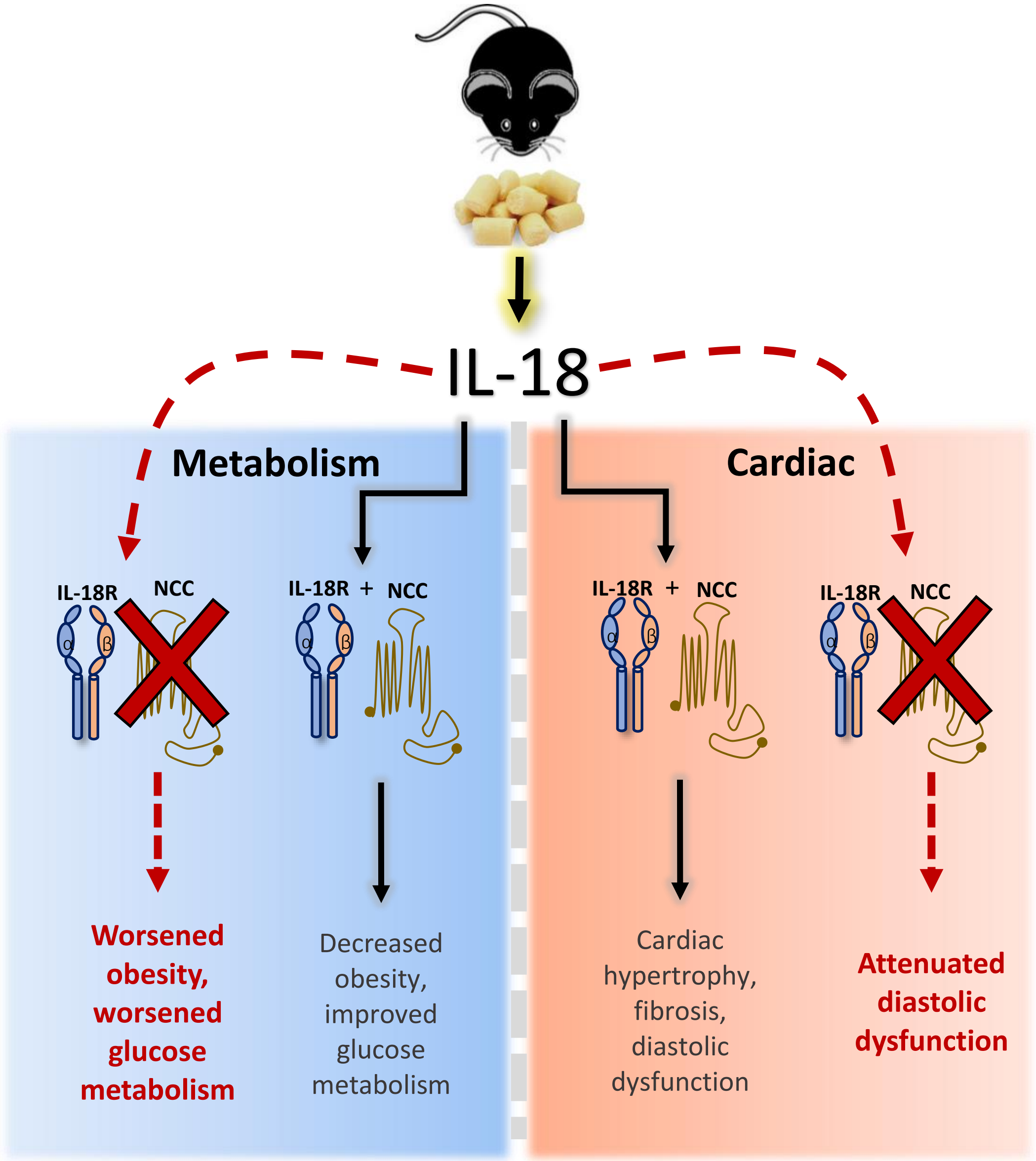
IL-18
Metabolism
Cardiac
IL-18R
NCC
α
β
IL-18R + NCC
IL-18R + NCC
IL-18R
NCC
Worsened obesity, worsened glucose metabolism
Decreased obesity, improved glucose metabolism
Cardiac hypertrophy, fibrosis, diastolic dysfunction
Attenuated diastolic dysfunction

Figure 13. Hypothesis Schematic.

Methods

a. Animals

i. All animal experiments were conducted under guidelines on the humane use and care of laboratory animals for biomedical research published by the National Institutes of Health. The Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University approved this study. Eight- to 12-week-old mice of the C57BL/6J background genetically modified to lack the gene for the Na-Cl Co-transporter (NCC) (NCC KO) and IL-18KO mice were purchased from The Jackson Laboratory (Bar, Harbor, ME).

b. Study Design

i. Prior to diet intervention, NCCKO male (n=10) mice had free access to standard chow and water. After baseline metabolic and cardiac assessment, each group was divided so that half of the group (n=5) was assigned to maintain a standard chow diet (Teklad LM-485; Envigo) while the other half (n=5) was assigned to maintain a “western diet” (WD) rich in saturated fat and sugar (Teklad TD.88137; Envigo). A group of female mice were studied as well to see differences associated with e gender. However, in this study, only the male mice are reported. IL-18 KO and wild-type (n=10/group) were also fed with WD to be used as comparison for the NCCKO mice. The study was performed over an 8 to 16 week period to assess metabolic and cardiac phenotype. Nutrient composition of each diet is listed in Table 2 below.

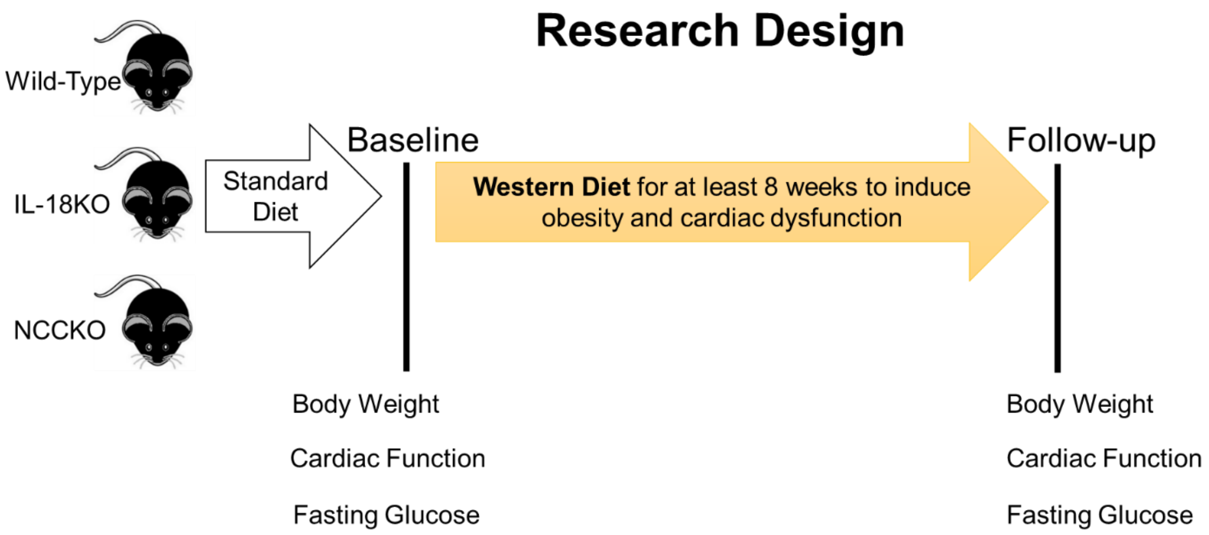
Research Design
Wild-Type
IL-18KO
NCCKO
Standard Diet
Baseline
Western Diet for at least 8 weeks to induce obesity and cardiac dysfunction
Follow-up
Body Weight
Cardiac Function
Fasting Glucose
Body Weight
Cardiac Function
Fasting Glucose

Figure 14. Research Design.

Dietary composition in %kcal	Standard Diet (SD)	Western Diet (WD)
Carbohydrate	44.3	42.7
Protein	19.1	15.2
Total Fat	5.8	42.0
Saturated Fat % of total fatty acids	0.8	61.8
Cholesterol	--	0.2
Kcal/g (Energy Density)	3.1	4.5

Table 2. WD Composition. Nutrient composition of Standard Diet (SD) and Western Diet (WD).

c. Food intake and body weight

i. Food intake was measured biweekly as the disappearance of food in grams (g) since last observation. Body weight was measured weekly using a scale and recorded in grams.

d. Oral Glucose Tolerance Test (OGTT)

i. Mice were fasted overnight (16 hours) before measurements of fasting blood glucose. The mice were transferred from their previous cage to clean cages with no evidence of food or feces in the new cage. Mice had free access to water during the fasting period. The blood was collected from the distal tails of the mice. The first drop of blood removed via gauze pad, and the following drop of blood was used to obtain serum glucose concentrations with an AimStrip *Plus* Blood Glucose Meter (Ermaine Laboratories Inc.) and AimStrip *Plus* Blood Glucose Test Strips (Ermaine Laboratories Inc.). Blood glucose concentrations were measured at baseline after the 16 hours fasting and at time points 15, 30, 60, and 120 minutes after glucose challenge. A 20% by weight D-(+)-Glucose (Sigma-Aldrich) solution in water (ex. 2g D-(+)-Glucose in 10mL water) and mice were carefully given 10µL/g body weight via oral gavage. All the NCC KO mice received the test at baseline and before the study termination, while the IL-18 KO and wild-type received the test only at baseline, and fasting glycemia prior to termination. For consistency, the fasting glycemia is reported for all three groups.

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e. Insulin Tolerance Test (ITT)

i. Mice were fasted for 6 hours before measurements of serum glucose. The mice were transferred from their previous cage to clean cages with no evidence of food or feces in the new cage. Mice had free access to water during the fasting period. Blood was collected from the distal tails of the mice, the first drop of blood removed via gauze pad, and the following drop of blood was used to obtain serum glucose concentrations with an AimStrip *Plus* Blood Glucose Meter (Ermaine Laboratories Inc.) and AimStrip *Plus* Blood Glucose Test Strips (Ermaine Laboratories Inc.). Serum glucose concentrations were measured at baseline after the 6 hours fasting and at time points 15, 30, 60, and 120 minutes after insulin challenge. Stock Insulin (Lantus, insulin glargine injection, provided by Virginia Commonwealth Division of Animal Resources) provided was 1 Unit/10µL and the overall administration was to be 1 Unit Insulin/1kg body weight of mice. The body weight of mice undergoing testing was summed to find the amount of Insulin Units needed (ex. 20 mice totaling 640g = .64 Units = 6.4µL stock Insulin). Insulin was diluted in 0.9% Normal Saline (NS) to make the final solution (ex. 20 mice totaling 640g requires 6.4mL final volume. For simplicity, a solution containing 10uL stock Insulin within a total 10mL volume with 0.9% NS as solvent was made). Mice were given 10µL/g body weight intraperitoneal injection of the Insulin/Saline solution and blood glucose was measured at time points mentioned above. This test was performed on NCC mice only, therefore the results are not part of the dissertation.

f. Echocardiography

i. Mice underwent transthoracic Doppler echocardiography (Vevo770; VisualSonic, Toronto, ON, Canada, 30MHz probe) under sedation (30-50 mgkg^{-1} pentobarbital) to evaluate systolic and diastolic parameters at baseline prior to diet administration, 4, 8, and 16 weeks after the beginning of either standard or western diet. B-Mode was used to find mid-papillary region of the left ventricle. M-Mode was then used to measure left ventricular end-diastolic diameter (LVEDD) and left-ventricular end systolic diameter (LVESD), then left ventricle fractional shortening (LVFS) was calculated [(LVEDD-LVESD)/LVEDD]*100. Pulse-Wave Doppler was used to assess isovolumetric contraction time (ICT), ejection time (ET), and isovolumetric relaxation time (IRT). Myocardial Performance index (MPI), also known as Tei index, an assessment of global systolic and diastolic function, was calculated as [(ICT+IRT)/ET].

g. IL-18 ELISA

i. Blood was collected from mice through the inferior vena cava at the time of death. Blood was incubated in tubes with heparin (BD, Franklin Lakes, NJ) for 15 min and then centrifuged at 2,000 rpm at 4°C for 10 min to obtain plasma. Samples were stored at −80°C and subsequently analyzed with a specific ELISA for murine IL-18 (plasma, MBL) according to the supplier's instructions to assess the induction of IL-18 after WD. Absorbance was read with a Bio-Tek plate reader (model µQuant, Bio-Tek, Winooski, VT) at 450 nm. Data are expressed in picograms per milliliter (pg/mL).

h. Statistical Analysis

i. For the animal study, because of the low expected variance within the groups, values are expressed as mean and SEM. The differences between groups were assessed using analysis of variance followed by the Student t test for unpaired data to compare the individual groups. Microsoft Excel and GraphPad Prism 7 were used for statistical analyses.

Results:

We set out to evaluate paramters of metabolism and cardiac function in three groups of mice fed a high-saturated fat diet: Wild-type, IL-18KO, and NCCKO.

WD induces systemic release of active IL-18

To assess the inflammatory state after WD, we measured plasma IL-18 in NCCKO and wild-type mice on WD and compared them to separate C57BL6/J wild-type mice that were on standard diet (SD). We observed an statistically significant increase in plasma IL-18 after WD in wild-type (183.3pg/mL ± 29.3) and NCCKO (237.6pg/mL ± 12.7) mice compared to wild-type mice on SD (79.3pg/mL ± 19.9) (Figure 15).

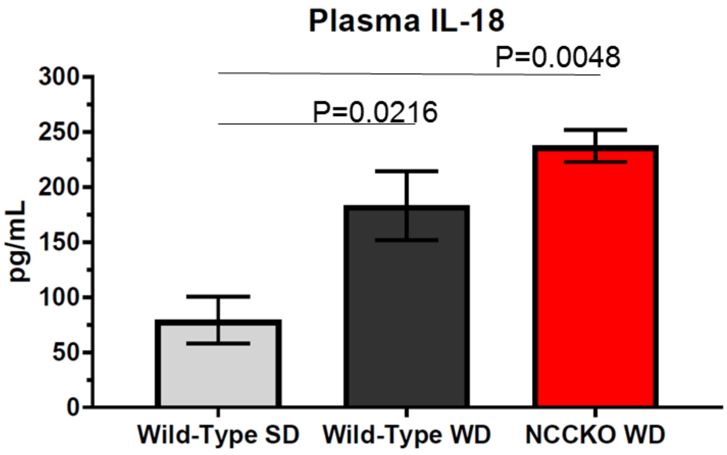

Figure 15. WD Increases circulating IL-18. Plasma IL-18 measured by ELISA in wild-type mice and NCCKO mice on WD compared to wild-type mice on SD (n=5-8). Data (in pg/mL) are reported as means ± SE (One-Way ANOVA — P-values vs. wild-type mice on standard diet).

Body Weight changes over study duration

Plotted in Figure 16 (top), we analyzed absolute body weight (g) at 1 week time points over the course of 8 weeks for Wild-type, IL-18KO, and NCCKO male mice. All the mice displayed normal appearance and behaviour. At baseline, IL-18KO mice weighed significantly more than NCCKO, but not more than wild-type mice. At the later time points, the IL-18KO mice started to weigh signifincantly more than the wild-type and maintained a constant difference from the NCCKO (Figure 16, top).

To evaluate rate of growth on WD relative to initial weight in all mouse strains, we calculated percent change from baseline (%) at each week (Figure 16, bottom). In this way, we can compare the rate at which the mice gain weight, independent from the differences at baseline. We found that the IL18KO and NCCKO gain a significantly larger percentage of weight, 46.4 % ± 4.1 and 55.0% ± 2.1 respectively, than the wild-type mice who gained 21.7% ± 6.8 of initial weight ($P<0.05$) (Figure 16, bottom). This data suggests that NCCKO mice on WD gain weight to a greater extent than wild-type mice that resembles body weight changes observed in IL-18KO mice on WD.

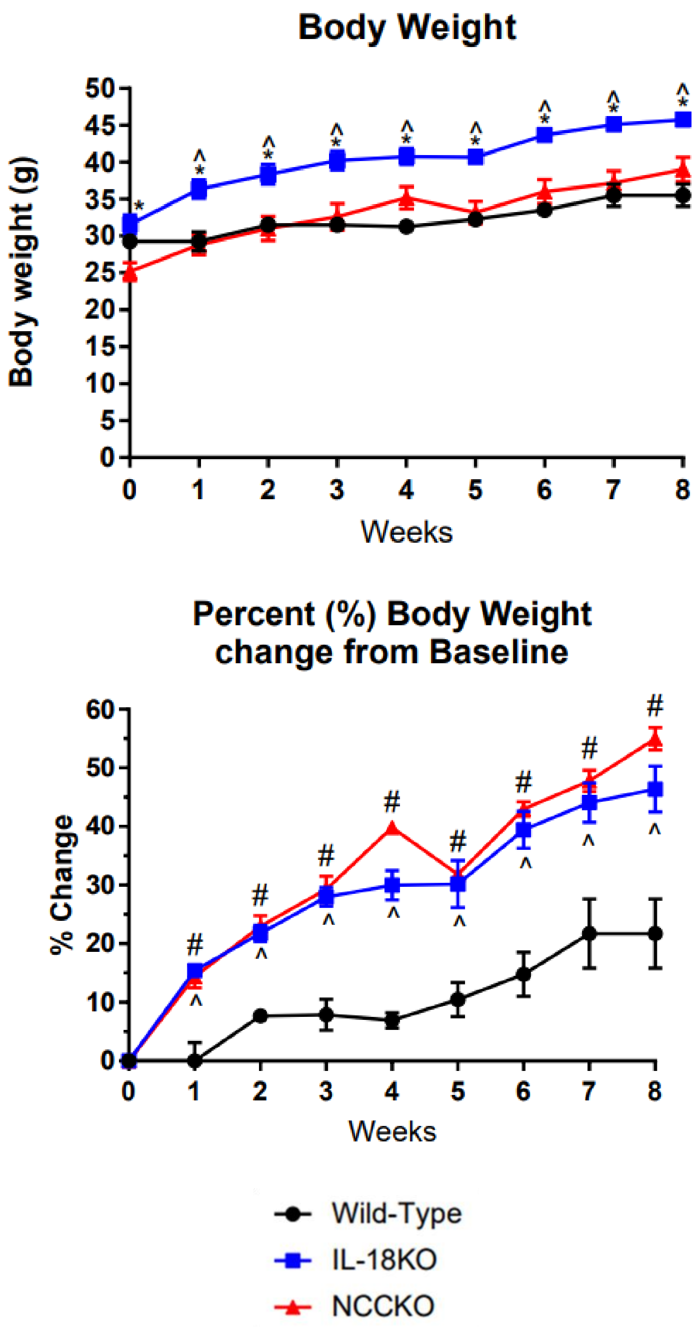

Figure 16. Increased Body Weight Gain of IL-18KO and NCCKO mice on WD. Absolute body weight gain (top) and percent body weight change from baseline (%) (bottom) comparisons between wild-type, IL-18KO, and NCCKO over 8 weeks while on Western Diet (n=5-9). (Two-Way ANOVA — #: P<0.05 NCCKO vs. Wild-type, ^:P<0.05 IL-18KO vs. Wild-Type, *:P<0.05 IL-18KO vs. NCCKO). Data (in g) are reported as mean ±SE.

WD induced Type II Diabetic phenotype

In order to assess metabolic function in the three groups of mice before and after WD consumption, fasting glucose levels were taken. At baseline before WD, IL-18KO and NCCKO mice had significantly higher fasting plasma glucose levels (93.3 ± 4.4 and 92.6mg/dL ± 5.6 respectively) compared to wild-type mice (78.1mg/dL ±1.7) (Figure 17 left, P<0.05). After WD, the fasting plasma glucose was not different between any groups (151.9 ± 10.6 vs. 165.4 ± 4.7 vs. 156.4 ± 8.0 mg/dL for IL-18KO, NCCKO, and Wild-Type respectively, Figure 17 right).

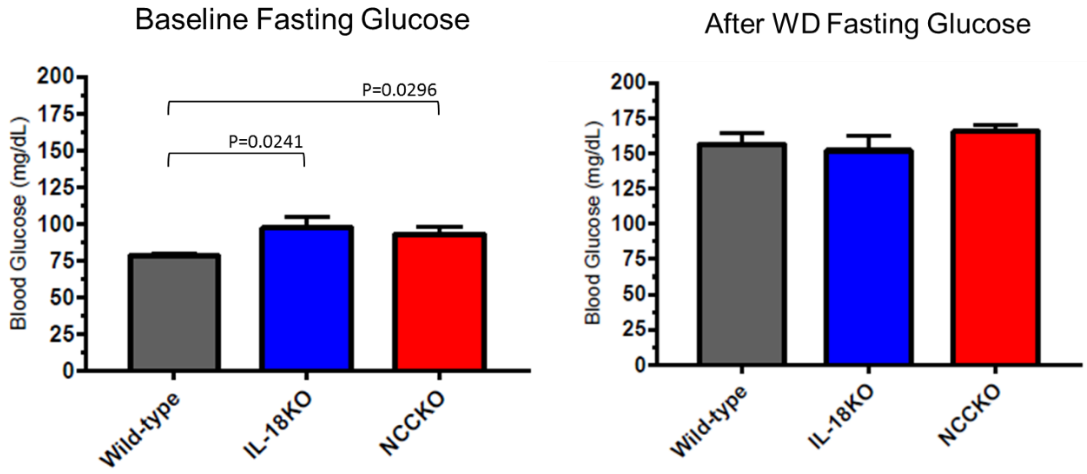

Figure 17. Differences in fasting glycemia before, but not after WD. Fasting blood glucose prior to western diet (baseline, left) and after 8 weeks (wild-type, IL-18KO) or 16 weeks of western diet (NCCKO) (right) (One-Way ANOVA, P-values vs. Wild-Type). Data (in mg/dL) are reported as mean ±SE, n=3-10.

Echocardiographic assessment of systolic, diastolic, and global function

To evaluate the change in cardiac function in the three groups after WD, we chose to evaluate percent (%) change from baseline in Fractional Shortening (FS), Isovolumetric Relaxation Time (IRT), and Tei Index. When evaluating FS, NCCKO mice had a 14% ± 8 decrease in FS, while IL-18KO and wild-type had a 13% ± 4, and 28% ± 5 decrease, respectively (Figure 18, top). The percent decrease from baseline was significantly less in the IL-18KO group compared to Wild-Type, but not statistically different between NCCKO and Wild-Type mice. NCCKO mice after WD had a significantly smaller increase (29% ± 16) in IRT compared to wild-type (123% ± 21), while the IL-18KO % increase in IRT (67 ± 26) was not statistically significant from wild-type (Figure 18, middle). The percent change in Tei index, a marker of global function, from baseline was significantly preserved in NCCKO and IL-18KO mice (62% ± 21 and 46 ± 22 increase respectively) compared to the wild-type percent increase from baseline (162% ± 23 increase, Figure 18, bottom). Collectively, these echocardiographic changes over time indicate that after WD, NCCKO mice exhibited less worsening of diastolic and global function compared to wild-type mice that resembled the IL-18KO phenotype.

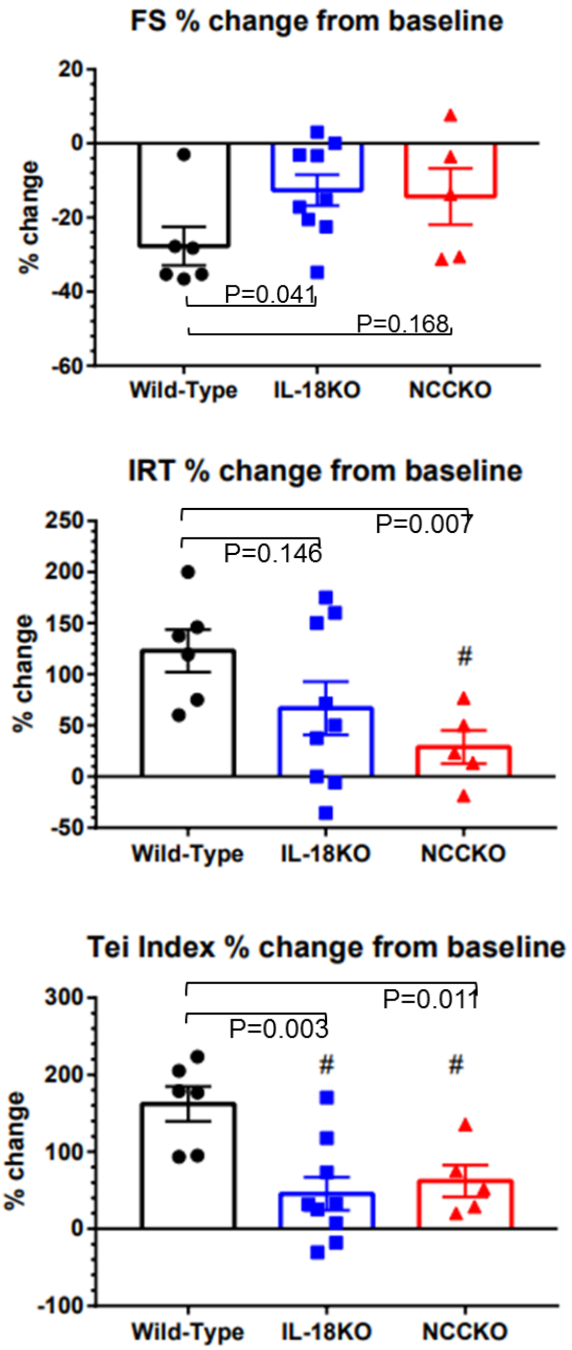

Figure 18. Attenuated changes in cardiac function after WD in IL-18KO and NCCKO mice. Echocardiographic measurements Fractional Shortening (FS, top), Isovolumetric Relaxation Time (IRT, middle), and Tei Index (bottom), percent changes from baseline to after western diet completion (wild-type and IL-18KO 8 weeks, NCCKO 16 weeks). One-Way ANOVA test to evaluate trend within groups, and T-test to evaluate statistical differences between groups: #:P<0.05 vs. Wild-Type. Data (in % change) are presented as mean ±SE, n=5-9.

Discussion

The results of the current study show that mice genetically lacking the Na-Cl Co-transporter have an overlapping phenotype with mice genetically lacking IL-18. IL-18 has both cardiodepressant activity and prevents weight gain by suppressing appetite. NCC is a newly discovered receptor for Interleukin-18, and we hypothesized that it may in part mediate the *negative* effects of IL-18 on cardiac function and the *positive* effects of IL-18 on metabolic function during WD feeding. After establishing that a WD induces increased levels of IL-18 in both wild-type and NCCKO mice (Figure 15), we assessed metabolic and cardiac phenotypes.

To study the role of NCC in metabolism, we used an accepted model of diet-induced obesity whereby mice are fed with a specific diet for at least 8 weeks to induce metabolic and cardiac dysfunction. The high energy density and high percentage of total and saturated fat diet used as WD (Western Diet) is advantageous in this basic science investigation due to its high relevance to consumption habits in western populations. NCCKO mice appear to be smaller than the IL-18KO mice at any weekly interval. However, to better evaluate weight gain while on the diet, the weight gain was measured and normalized by the initial weight (Figure 16, bottom). Evidently, the rate of body weight increases from baseline of the NCCKO mice on WD (55% at 8 weeks) has striking resemblance to the rate of body weight increase of IL-18KO mice on WD (46.4% at 8 weeks) where both groups gained significantly more weight relative to baseline than wild-type mice on WD (21.7% at 8 weeks). This is of importance when compared to available literature that has investigated body weight gain of IL-18RαKO mice. Two groups have observed a pattern whereby the IL-18RαKO mice growth rate on a WD is slower than that of IL-18KO and more closely resembles that of a wild-type mouse on WD (Figure 10, Introduction) ([102,104]). The trend of acute onset and sustained obesity while on WD in mice lacking IL-18 or an upstream activator such as NLRP1 or TLR4 is well-documented in the literature ([98,99,104,108]).

Our observations indicate that perhaps during a state of high-saturated fat feeding which induces a low-grade pro-inflammatory state, IL-18 may be signaling in the brain to control appetite and in the heart to regulate relaxation to a greater degree through NCC rather than the IL-18Rα. Support for IL-18 “favoritism” to NCC has been observed *in vitro*. Conjugated IL-18 showed a higher binding affinity to NCC than to IL-18Rα [NCC dissociation constant (a concentration that indicates when 50% of binding has occurred) K_d = 17.3nM vs. IL-18Rα K_d= 46nM] ([51,105]). Additionally, IL-18 has been shown to increase peritoneal macrophage NCC mRNA levels by 65 fold, as well as increase plasma membrane NCC in macrophages and T-cells treated with IL-18, observable by immunostaining ([105]). It is possible, but has not been analyzed to our knowledge, that IL-18 may cause a similar increase in NCC within neural tissue that becomes primarily responsible for binding to IL-18 and modifying neural circuits to decrease satiety and eventual body weight.

Importantly, this hypothesis would necessitate careful investigation of food intake. A common method of measuring food intake is to measure the disappearance of food from the mice cages; however, this measurement has high variability due to the fact that mice “play with” the sticky texture of the high-saturated fat diet and do not necessarily ingest all of the WD that disappeared from the previous measurement. This method was attempted with these groups of mice, but the pitfall of the measurement tool limited the accuracy of the data. A more precise method of measuring food intake using a metabolic chamber is in planning to best evaluate physiologic changes in food intake in similar groups studied here.

It cannot be understated that a key component of IL-18 signaling in *positively* affecting metabolism is within the central nervous system (CNS) to decrease appetite and food intake. Common to pro-inflammatory states of non-specific illness is the loss of appetite([109]). Interleukin-18 has been identified in the mouse to contribute to this phenomenon. Through a pre-synaptic disinhibitory mechanism, IL-18 disrupts the balance of excitatory and inhibitory electrical signals in the amygdala that culminates in increased lateral hypothalamic (LH) firing and a decrease in food intake ([101]). An observed reduction in standard diet food intake after central IL-18 administration was deemed to be dependent on the presence of the IL-18Rα as brain slices from IL-18RαKO mice treated with rIL-18 did not exhibit significant changes in electrical properties changes as seen in brain slices from IL-18Rα$^{+/+}$ mice. However, these experiments were done in IL-18RαKO mice 3-5 months of age on a *standard diet*. Mice on a standard diet have been shown to have significantly less (approximately 30%) plasma IL-18 than mice fed a WD for 8 weeks ([86]). Additionally, the ELISA results shown here indicate circulating IL-18 approximately doubles after WD (Figure 14). It is possible the IL-18Rα$^{+/+}$ and IL-18RαKO mice fed standard diet, and studied specifically for IL-18/IL-18Rα mediated hypophagia, did not have sufficiently elevated IL-18 levels to induce relevant NCC expression within the amygdala and hypothalamus, therefore NCC function as a receptor could not have been evaluated in that setting.

Additionally, our analysis of the obese phenotype through weight gain is not as holistic as appears in other literature that separates body composition by fat mass and fat-free mass with the use of sophisticated tools such as dual energy X-ray absorptiometry (DEXA) or EchoMRI. A possible relevancy of accurately measuring body mass composition to the NCCKO mice here is a finding of increased bone mineral density (BMD) in NCCKO mice compared to $NCC^{+/+}$ mice. However, despite an increased BMD, the absolute weight of littermate $NCC^{-/-}$ and $NCC^{+/+}$ mice was indifferent (25.7g vs. 25.9g respectively) ([110]). More specific tools, while more expensive, could have elucidated more intricacies of fat and lean mass within the obese phenotypes observed.

To assess whole body glucose metabolism we measured overnight fasting plasma glucose levels before and after WD. Prior to the start of WD the IL-18KO and NCCKO mice had significantly higher fasting glucose (93.3 and 92.6mg/dL respectively) compared to wild-type mice (78.1mg/dL) (Figure 17, left). Although these levels are still within a normal range, they indicate that IL-18 and NCC are directly or indirectly important for glucose homeostasis. Similar observations have been identified for IL-18KO mice where, by 3 months of age IL-18KO mice have elevated fasting insulin and decreased tolerance to glucose and by 6 months of age exhibit full-fledged Type II Diabetes characterized by elevated fasting glucose, decreased glucose tolerance, and decreased insulin sensitivity ([96]).

Peripheral homeostatic functions of IL-18 that relate to energy metabolism are not well understood. In patients, elevated plasma IL-18 are positively associated with BMI, Type II Diabetes (T2D), and waist-to-hip ratios, and negatively correlated with insulin sensitivity (93 111). This could reflect another phenomenon seen in obese patients of IL-18 resistance. Leukocytes isolated from obese or T2D patients have a blunted production of INF-γ in response to IL-18 stimulation that was accompanied by a 50% reduction in plasma membrane Il-18Rα and IL-18Rβ (112). Interestingly though, intraperitoneal recombinant IL-18 was able to improve insulin sensitivity in IL-18$^{+/+}$ and IL-18$^{-/-}$ mice (96). Additionally, *in vitro* and *ex vivo* studies have shown that exogenous IL-18 treatment increases AMPK and fat oxidation (102). AMPK is an intracellular protein that coordinates a multitude of cellular functions, including fatty acid uptake, β-oxidation, translocation of GLUT-4 vesicles to the plasma membrane, promotion of glycolysis and inhibition of gluconeogenesis, providing further support for IL-18 acting in part to improve glucose metabolism (113).

Although no other basic science studies to our knowledge have investigated the effects of NCCKO on glucose metabolism, two potential clinical correlations can be made. A possible link that has been made for the development of glucose intolerance and insulin resistance in patients with genetic mutations in NCC, clinically named Gitelman Syndrome, or hypokalemia in patients receiving thiazide diuretics (hydrochlorothiazide, chlorthalidone: NCC blockers). Gitelman Syndrome patients and patients on thiazide diuretics may exhibit hypokalemia owing to increased urinary K+ excretion in the distal tubule and collecting duct (114). Thiazide-induced dysglycemia is a well-observed phenomenon, but the mechanism behind it is unclear. Speculation to this point focuses on K+ mediated release of insulin by pancreatic β-cells, but it is not established as to whether small decreases in K+ are physiologically relevant to cause a decrease in insulin secretion that results in hyperglycemia (115).

Connecting apparent findings of IL-18 *improving* insulin sensitivity, and NCC acting as a receptor for IL-18 binding and effects, it is possible that IL-18 functions through NCC to improve insulin sensitivity, although this requires much further investigation. Although we see higher fasting plasma glucose at baseline in the NCCKO and IL-18KO compared to wild-type, WD over time does not worsen glycemia, indicating glucose metabolism during high-fat diet may be independent of IL-18 and NCC function (Figure 17, right). This finding is somewhat dissociated from the severity of obesity development. Despite gaining significantly more weight than wild-type, NCCKO and IL-18KO display similar fasting glycemia. Future investigation into glucose metabolism and insulin function is of interest in the NCCKO genotype to perhaps investigate contributions of electrolyte abnormalities and/or IL-18 mediated effects to alterations in glucose metabolism.

To evaluate the contribution of NCC to cardiac function during WD, we assessed systolic, diastolic, and global function parameters via echocardiography. We report percent change in cardiac function of groups to normalize for baseline differences and specifically evaluate the effect of the WD on changes in cardiac function. For all three groups there was decline in fractional shortening (FS) with only the IL-18KO group having less worse FS changes after WD compared to wild-type mice (13% vs 28% decline respectively) (Figure 18, top), indicating systolic function was not statistically preserved by genetic deletion of NCC after WD. This result was somewhat expected as the model of diet induced obesity induces mild systolic dysfunction, but may retain an ejection fraction that is still considered preserved.

Pulse wave Doppler measurements allowed for analysis of diastolic and global function, specifically isovolumetric relaxation time (IRT) and Tei Index (or Myocardial Performance Index). For orientation, an increase in IRT or Tei Index indicates worsening function. In terms of changes in the early relaxation of the left ventricle after WD, NCCKO mice had significantly attenuated percent changes from baseline compared to Wild-Type (29% vs. 123%), whereas the IL-18KO percent change from baseline was not significantly less than the Wild-Type (67% vs. 123%) (Figure 18, middle). Further support for a less severe effect of WD on cardiac function for NCCKO mice is supported by a statistically lower percent increase from baseline in global evaluation via the Tei Index. After WD, both the IL-18KO and NCCKO mice had significantly lower percent increases in Tei Index (46% and 62% increase respectively) compared to wild-type mice (162% increase), indicating that the WD had less of a cardiodepressant effect in the IL-18KO and NCCKO groups (Figure 18, bottom). Collectively, the echocardiographic results indicated that IL-18 induced by WD may have a greater impact on diastolic function, and the genetic knockout of IL-18 or the NCC receptor for IL-18 resulted in less worsened diastolic dysfunction from baseline after WD.

Keeping in mind clinical heart failure relevancies, a large clinical study evaluating efficacy of differing anti-hypertensive pharmacotherapy in high-risk hypertensive patients discovered that the use of a NCC blocker, Chlorthalidone, significantly reduced the risk of heart failure at 4-8 year follow up compared to an ACE inhibitor (Lisinopril) or a Calcium-Channel Blocker (Amlodipine) ([107]). This finding represents a possible link to the research investigated here, whereby inhibition of NCC aside from the primary goal of diuresis to control blood pressure may also be provide direct inhibition of IL-18 signaling in the myocardium to prevent or slow the progression of cardiac dysfunction. This line of thinking is of high interest that we aim to investigate further in the future.

An alternative method to evaluate novel contributions of NCC to IL-18 signaling could have been through the use of pharmacologic inhibitors of the ion transporter that are commercially available, notably hydrochlorothiazide (HCTZ). A flaw to this design are reports that HCTZ does not cross the blood brain barrier and therefore centrally mediated effects of NCC may not be able to be assessed; however, this could have been acutely overcome with direct intracerebral administration of HCTZ via cannulas placed into the skull and brain ([120]). This direct central administration method is complicated; however, by post-surgical induction of pain, distress, and risk for infection ([121]). Additionally, although the genetic knockout of NCC has yet to result in embryonic lethality to our knowledge, NCC has important other physiologic contributions. As noted prior, the Na-Cl Co-transporter is partially responsible for electrolyte homeostasis, particularly K^+, Mg^{2+}. Therefore, a temporally controlled inducible mutation of *SLC12a3*, the gene encoding NCC, in mice could prove to be a beneficial model to study the physiology related to NCC in the future.

A separate limitation of this study is the fact that the wild-type mice are not littermates to the NCCKO or IL-18KO mice. Although all of the mice studied have the same C57Bl6/J background, this is of great importance when attempting to control for a single genetic change and we cannot exclude the effects of other compounding genetic alterations when assessing the data. Moreover, genetic knockout mice remain inbred, which can lead to reduced biological fitness of the population.

The strength of research presented here derives from the novel findings, specifically regarding the possible role of the Na-Cl Co-transporter (NCC) in mediating IL-18 effects on metabolic and cardiac function. We believe the investigation and results here have laid a foundation for a more elaborate research plan designed to comprehensively investigate the known components of IL-18 signaling in a similar experimental model. Others have described that protection from the pro-inflammatory disease of atherosclerosis is observed only after combined genetic knockout of IL-18R and NCC ($IL\text{-}18R\alpha^{-/-}NCC^{-/-}$), and not singular littermate knockouts of either ($IL\text{-}18R^{-/-}$ or $NCC^{-/-}$) (105). Although the data presented here investigates the sole knockout of NCC, in the future we hope to replicate the double knockout design and further investigate IL-18 signaling mechanisms in the context of diet-induced obesity. In order to accomplish this, crossbreeding to obtain littermates with differing components of the IL-18 signaling system missing must be done. Crossbreeding and genomic analysis is currently underway to establish these mouse lines in preparation for future studies.

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