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Effects of Exercise on Epigenetic Pathways in Persons with Heart Failure

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Effects of Exercise on Epigenetic Pathways in Persons with Heart Failure

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Abstract

Effects of Exercise on Epigenetic Pathways in Persons with Heart Failure
By Brittany Butts

Introduction: Inflammation contributes to heart failure (HF) progression and interleukin (IL)-1 cytokines IL-1β and IL-18 are implicated in this process. The adaptor protein ASC (apoptosis associated speck-like protein containing a caspase recruitment domain) is necessary for inflammasome activation of IL-1β and IL-18. Lower ASC methylation is associated with worse outcomes in HF. The purpose of this study was to examine the effects of exercise on changes in ASC methylation and activation of interleukin-1 family cytokines IL-1β and IL-18 in persons with HF.

Methods: Participants (N=54) were randomized to receive exercise intervention (n=38) or attention control (n=16) for 3 months and were followed for an additional 3 months post-intervention. Blood samples for measures of percent methylation of the ASC gene, plasma IL-1β, IL-18, and ASC mRNA were obtained at baseline, 3 months, and 6 months.

Results: ASC methylation was higher in the exercise group as compared to control at 3 months (6.10±0.5% vs. 5.80±0.4%; p=.04) and 6 months (6.07±0.4 vs. 5.82±0.4; p=.04). Plasma IL-1β was lower in the exercise group at 3 months (1.43±0.5 pg/mL vs. 2.09±1.3 pg/mL; p=.02) and 6 months (1.49±0.5 pg/mL vs. 2.13±1.4 pg/mL; p=.004). In the exercise group, ASC methylation was higher at 3 months as compared to baseline (p=.009), and IL-1β was lower than baseline at both 3 (p<.001) and 6 months (p=.04). ASC mRNA expression was negatively associated with ASC methylation at baseline (r=-.97, p=.001), 3 months (r=-.90, p=.001), and 6 months (r=-.81, p=.001). ASC mRNA was lower than baseline at 3 months (p=.004) and 6 months (p=.002) among those in the exercise group. Significant group differences in change scores from baseline to 3 months were found for IL-1β (t=3.73, p=.001) and ASC methylation (t=-2.71, p=.01).

Conclusions: Exercise was related to increased mean percent ASC methylation and decreased IL-1β and ASC mRNA gene expression in HF. Epigenetic regulation of ASC may be a biological mechanism by which exercise can promote better outcomes in HF. Further research examining mechanisms of change can lead to improved understanding of physiological adaptations and more precise prediction of adverse outcomes in persons with HF.

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CHAPTER I

INTRODUCTION

Statement of the Problem

The syndrome of heart failure (HF) results from various structural or functional impairments in cardiac function leading to an inability to maintain normal cardiac output at normal filling pressures.[1,2] HF remains a major cause of morbidity and mortality in the United States and is the leading cause of hospitalization among individuals over age 65 years, leading to current costs of care exceeding 34 billion dollars annually.[3] Over the past two decades, advances in pharmacological and device therapies for HF have significantly improved prognosis for HF patients with low ejection fraction. However, the overall prognosis continues to be poor for these patients with mortality rates approaching 50% in 5 years.[4] Therefore, attenuating HF disease progression remains an important research and clinical goal. Identification of novel pathways and effectively intervening on potential therapeutic targets may slow disease progression in HF.

It is known that HF is associated with a chronic low-grade inflammation leading to adverse cardiac remodeling and disease progression.[5] This chronic inflammation is characterized by the formation and activation of an intracellular protein complex, the inflammasome, which in turn activates inflammatory cytokines that promote cardiac hypertrophy and myocardial apoptosis.[5-7] Increased inflammatory cytokines are associated with increased HF progression, severity, and death.[1,8-10] IL-1β and IL-18 are pleiotropic, inflammatory cytokines theorized to play a prognostic and mechanistic role in HF;[1,10-12] increased levels have been shown to contribute significantly to worsening HF

severity and mortality.[1,8,9] IL-1β and IL-18 increase during acute HF decompensation, suggesting a role in myocardial dysfunction.[8] IL-18 induces the production of tumor necrosis factor-alpha (TNFα),[11] while IL-1β induces nitric oxide production, as evidenced by a corresponding increase in the production of inducible nitric oxide synthase (iNOS), leading to worsening of ventricular remodeling in HF.[13]

IL-1β is expressed as proform 1L-1β upon immune activation while IL-18 is constitutively expressed as proform IL-18; both molecules require caspase-1 dependent proteolytic cleavage for activation.[12] Caspase-1 is recruited by an adaptor molecule, ASC, which leads to IL-1β and IL-18 activation.[13] ASC expression is epigenetically controlled by CpG methylation.[14] Increased methylation inversely correlates with ASC expression, ASC methylation has been shown to increase with aerobic exercise.[14]

The 2013 American Heart Association recommendations for HF include aerobic exercise (AEX) as a safe and effective non-pharmacological therapy that improves physical and psychological function,[4] reduces hospital readmission rates, lowers mortality in some studies and improves quality of life.[15-17] Chronic alterations in myocardial load are characteristic of HF and lead to ventricular remodeling, a strong prognostic indicator of adverse clinical outcomes. Proposed mechanisms of abnormal ventricular remodeling in HF center on inflammatory and oxidative processes.[8,18,19] AEX has been shown to partially reverse some of these mechanisms and improve clinical outcomes [17,20,21], but a modifiable pathway has yet to be established in HF. This study proposed a pathway in which a short-term AEX intervention reduces IL-1β and IL-18 in HF and, in turn, reduces circulating TNF-alpha and iNOS, thus potentially improving outcomes.

Purpose

The purpose of this study was to examine the effects of aerobic exercise (AEX) on changes in ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) methylation and to determine whether this initiates a downstream change in inflammatory cytokines (interleukin-1β [IL-1β], interleukin-18 [IL-18]) known to worsen outcomes in persons with heart failure (HF). Because HF remains a leading cause of morbidity and mortality in the United States,[5,6] identification of novel therapeutic targets that may slow disease progression are urgently needed. Modification of the inflammasome has been proposed as a lower-risk alternative to current pharmacological trials targeting circulating cytokines, but only trials using murine models have been reported to date.[22] Therefore, this study proposed a non-pharmacological, modifiable molecular pathway, ASC methylation, as an aerobic exercise intervention target for persons with HF.

Specific Aims

Aim1: Examine the effects of AEX on ASC methylation in persons with heart failure.

H1: Persons with HF, who participate in a 3-month AEX intervention, will have increased ASC methylation at the completion of the intervention as compared to persons with HF in an attention-control group.

RQ1: Are intervention effects on ASC methylation sustained at 6 months, 3 months after completing the AEX intervention?

Aim2: Examine the relationship of ASC methylation and inflammatory cytokines in persons with HF.

H2: Increased ASC methylation will be related to decreased plasma IL-1β, IL-18, and TNFα and with decreased iNOS mRNA expression in persons with HF.

RQ2a: Does increased ASC methylation, in response to AEX intervention, target IL-18 activation, as evidenced by changes in the ratio of proform IL-18 mRNA to plasma IL-18?

RQ2b: Does increased ASC methylation have a *mediating* effect on changes in circulating IL-1β and IL-18 in persons with HF?

Exploratory Aim: Examine the relationships between percent ASC methylation, plasma IL-1β, and plasma IL-18 with aerobic capacity, as measured by peak VO_2, in persons with HF.

Conceptual Framework

The conceptual model for this study, portrayed in Figure 1.1, is the underlying physiological mechanisms of IL-1β and IL-18 activation, inflammation in HF and ASC methylation changes after aerobic exercise. This model outlines the pathway proposed in this study and ties together the different pieces of work previously established along the proposed pathway.

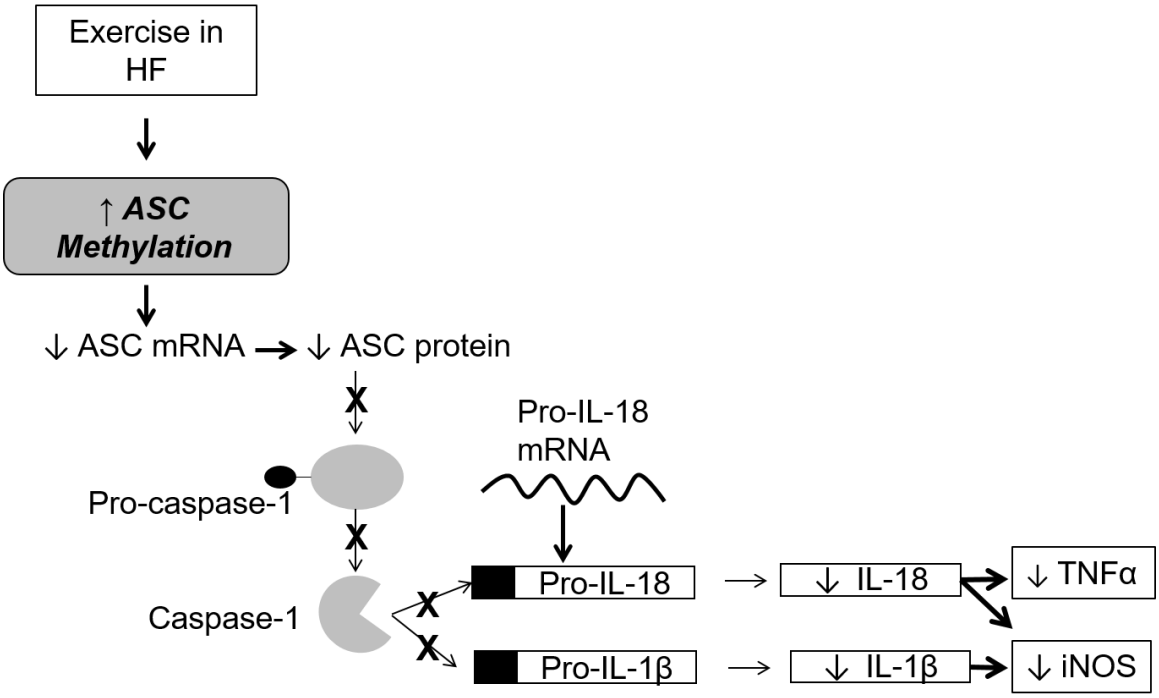

Figure 1.1 Conceptual Framework The conceptual model for this study is the underlying physiological mechanisms of IL-1β and IL-18 activation, inflammation in HF and ASC methylation changes after aerobic exercise. This model outlines the pathway proposed in this study and ties together the different pieces of work previously established along the proposed pathway.

Relevance of the Study

Inflammatory pathways and inflammasome modulation have been proposed as novel therapeutic targets in HF,[23] but few non-pharmacological treatments or interventions targeting a modifiable inflammatory pathway are currently proposed in HF. Further, epigenetic regulation of the inflammatory response has been identified as an important therapeutic approach to disease management,[24,25] but trials in persons with HF have not been reported to date. This study examines a biobehavioral-based non-

pharmacological intervention, aerobic exercise, as a method of epigenetic regulation of the inflammasome, a key mediator of inflammation in HF. This study examines aerobic exercise as an anti-inflammatory therapy in HF using inherent mechanisms of inflammasome modulation, and will bridge the gap between the current understanding of the role of exercise in immune responses and potential underlying mechanisms in HF.

BACKGROUND

Importance of Inflammation in Heart Failure

Several hypotheses have been generated in the search to explain the myocardial changes that lead to the progression of HF. Based on the etiology of HF symptoms, the "hemodynamic hypothesis" implicated hemodynamic stressors as the sole driver of HF progression.[26] However, studies using vasodilator drugs and positive inotropic drugs showed little support for hemodynamics as a driver of the myocardial remodeling leading to progression of HF.[27-29] Studies using angiotensin-converting enzyme (ACE) inhibitors, beta-adrenergic blocking drugs, and inotropic agents tested the "neurohormonal hypothesis", which states that heart failure progression is due to overexpression of endogenous neurohormones that chronically activate the sympathetic nervous system and renin-angiotensin system.[26] While the neurohormonal hypothesis did not provide a full explanation of HF progression, as evidenced by high HF morbidity and mortality despite ACE inhibitor and beta-blocker drug therapy, it demonstrates a shift in the way researchers thought about the mechanisms of disease. Research began to investigate how the cause of HF triggered endogenous mediators that lead to myocardial damage and loss of myocardial cells. Inflammatory cytokines, such as TNFα, IL-1, IL-6

and c-reactive protein (CRP), were found to be increased in those with HF. Further, levels of circulating cytokines were found to increase with HF severity and worsened prognosis.[30] Increased levels of circulating cytokines were hypothesized to lead to myocardial remodeling, cardiomyocyte hypertrophy and apoptosis, decreased myocardial contractility, myocardial fibrosis, and other structural changes that lead to HF progression.[20,31,32]

The consistent findings of increasing circulating inflammatory cytokines in worsening HF led to the "cytokine hypothesis" of HF.[30] The cytokine hypothesis states that high concentrations of circulating cytokines exert harmful effects on the heart, leading to myocardial remodeling and exacerbation of hemodynamic abnormalities.[30,32] Although some early findings suggested that inflammatory cytokines in HF are representative of a mere epiphenomenon, recent evidence is more suggestive of a mechanistic role in the disease process.[33] Initial studies of cytokines implicated in the pathogenesis of heart failure primarily focused on identifying individual cytokines, such as TNFα and IL-6. However, delving deeper to uncover the pathophysiological processes of myocardial remodeling that lead to the development and exacerbation of HF requires further study of the inflammatory pathways and underlying mechanisms of cytokine activation.

Danger-Associated Molecular Patterns (DAMPs)

Inflammation in HF is initiated by danger-associated molecular patterns (DAMPs), which are host-derived molecules indicative of cellular damage and has been shown to modulate irreversible myocardial changes, such as fibrotic changes,

cardiomyocyte apoptosis, and cardiomyocyte hypertrophy.[6,34,35] Proposed mechanisms of DAMP formation in HF include mitochondrial dysfunction, cellular death, ischemia, cardiac load and oxidative stress.[24,36-39] Mitochondrial dysfunction and necrotic or apoptotic cardiomyocyte death lead to the release of cellular components such as mitochondrial and nuclear nucleic acids, reactive oxygen species, N-formyl proteins, extracellular ATP, protein aggregates, and other debris.[24,36,40] Transient ischemia and reperfusion injury, under perfusion of myocardial tissue, and other sources of oxidative stress lead to the production of reactive oxygen and nitrogen products, which are powerful DAMPs associated with ventricular remodeling.[1,41] Increased ventricular filling pressures, cavity distension, systemic congestion, increased shear stress, and other chronic alterations in loading conditions lead to myocardial injury. Byproducts of this injury are detected by myocytes and immune cells as DAMPs and lead to an accelerated sterile inflammation in HF.[37,42] The inflammatory response amplifies the production of DAMPs, resulting in a positive-feedback loop accelerating HF pathophysiology.[24] Increased cardiac pressure and poor pump function directly trigger activation of inflammatory cells, such as peripheral monocytes, which aggregate in the heart and are released into circulation.[42,43] Activated inflammatory cells release pro-inflammatory cytokines, such as TNFα, which magnify the inflammatory process and contribute to fibrotic changes in the myocardium and progressive remodeling.[5,34,43,44]

NLRP3 Inflammasome

DAMP-activated inflammation occurs via the inflammasome, a complex of intracellular interaction proteins that recognizes DAMPs and triggers maturation of

inflammatory cytokines to initiate and amplify the inflammatory response.[35,44-46] The inflammasome is composed of a NOD (nucleotide-binding oligomerization domain)-like receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain, also known as PYCARD), and pro-caspase-1.[19,47,48] The activated inflammasome cleaves pro-caspase-1 into active caspase-1.[19,49] Caspase-1 in turn, activates the interleukin (IL)-1 family inflammatory cytokines IL-1β and IL-18, by cleavage of pro-IL-1β and pro-IL-18 into active forms.[12,14,50] Thus, the inflammasome is a powerful mediator of the immune response via caspase-1 activation of IL-1β and IL-18. The NLRP3 inflammasome can also induce pyroptosis, caspase-1-dependent apoptosis.[51] Loss of cardiomyocytes via pyroptosis reduces contractile reserve leading to HF progression.[52] In addition, as cytosolic components are released with pyroptosis, extracellular ASC becomes a danger signal and functions to initiate further inflammasome formation. ASC in extracellular space continues to activate caspase-1, propagating the inflammatory cascade.[52]

NLRP3 Inflammasome Activation

Both exogenous (pathogens, toxins) and endogenous (DAMPs) molecules activate the NLRP3 inflammasome.[19,53,54] Inflammasome formation and activation require two signals: a priming signal and an activating signal.[51] First, danger signals (DAMPs) activate the transcription factor NF-κB, leading to the production of NLRP3 and pro-IL-1β.[54] Other components of the NLRP3 inflammasome pathway (ASC, pro-caspase-1, and pro-IL-18) are readily available in steady state.[55] While the definitive mechanism of NLRP3 inflammasome activation has yet to be uncovered, proposed mechanisms include

cation movement (such as K^+ efflux or $Ca2^+$ influx), mitochondrial membrane dysfunction, frustrated phagocytosis, production of reactive oxygen species, and direct binding of oxidized mitochondrial DNA to NLRP3 itself.[51,53,55,56] This two-step process likely represents a regulatory checkpoint in inflammasome activation. Assembly of the inflammasome occurs when the N-terminal pyrin domain (PYD) of NLRP3 interacts with the PYD of ASC in a homotypic fashion, leading to recruitment and activation of caspase-1.[34,47,51,57] Further work is needed to elucidate mechanisms of inflammasome activation in HF.

NACHT, LRR, and PYD Domains-Containing Protein 3

NACHT, LRR, and PYD domains-containing protein 3 (NLRP3, aka NALP3 or cryopyrin) is a pattern recognition receptor (PRR) implicated in the pathogenesis of inflammation in chronic disease.[19] NLRP3 transcription is regulated by nuclear factor κB (NF-κB) in the presence of a danger signal, e.g. DAMPs. NLRP3 is made up of 3 functional domains: C-terminal leucine-rich repeats (LRR) with regulatory function, a NACHT domain that has ATPase activity, and PYD that serves as a death-fold domain.[19,47,49] NLRs are believed to play a role in the cytosol analogous to that of the toll-like receptors (TLRs) in the plasma membrane. Like other NLR family receptors, NLRP3 functions to guard the intracellular environment to maintain homeostasis.[58] NLRP3 differs from other nod-like receptors in that physiological expression levels are not sufficient for inflammasome activation. NLRP3 expression is up-regulated via NF-κB upon the sensing of a danger signal. NLRP3 functions as both an intracellular PRR, surveying the cytosol for danger signals, and as a platform protein in inflammasome

formation, initiating an inflammatory response.[58] NLRP3 is expressed in a number of cells, e.g. leukocytes, myocytes, and cardiac fibroblasts.[59] NLRP3 knockout mice have smaller areas of infarct in an experimental model of acute myocardial infarction;[35] constitutively active NLRP3 leads to uncontrolled activation of the inflammasome.[60] There is no evidence of transcriptional control of NLRP3 to date.

Apoptosis Associated Speck-Like Protein Containing a Caspase Recruitment Domain

ASC is a vital component of the inflammasome and functions to recruit caspase-1 to the inflammasome complex.[5,6,14,50] ASC is necessary for activation of pro-caspase-1 into caspase-1, which in turn is necessary for activation of IL-1 family cytokines, such as IL-1β and IL-18.[5,6] ASC deficiency lessens inflammatory response to ischemia-reperfusion injury in the myocardium.[34] ASC plays a vital role in the activation of the inflammasome and in the inflammatory response to danger signals. ASC expression is controlled through epigenetic modification via methylation. ASC methylation is inversely correlated with ASC protein expression and is silenced by overexpression of DNA methyltransferase.[14,61] Hypermethylation leads to an inactive state in which no ASC protein is expressed while complete demethylation induces cellular apoptosis via p53 and TRAK activation.[61] Thus, inflammasome formation and activation may be reduced via increased ASC methylation. Increased ASC expression corresponds to a decrease in methylation.[62] Moderate intensity aerobic exercise has been associated with increased ASC methylation in one study.[14] However, this study did not compare changes in ASC methylation before and after an exercise program but rather compared ASC methylation

of a group that had completed an exercise program to a control group. Further, this study did not compare changes in ASC methylation to ASC gene expression or circulating cytokines, such as IL-1β or IL-18. No studies have examined the effects of exercise on DNA methylation in persons with HF.

Caspase-1

Caspase-1 is a cysteine protease that functions as an effector protein in the NLRP3 inflammasome. Caspase-1 is produced by the cell as a zymogen, pro-caspase-1, which undergoes autocatalysis for activation upon a homotypic interaction with the CARD domain on ASC.[50,56] Activated caspase-1 is involved in the recruitment of innate immune cells to sites of inflammation, induces pyroptosis, and is the primary activator of IL-1β and IL-18.

Attempts to Modulate the Inflammasome

Strategies to alter inflammasome function in mice have included: genetic knockouts of inflammasome proteins and interleukin-1β; binding circulating proteins (e.g., IL-1 Trap); IL-1 receptor antagonists (e.g., anakinra); and siRNA silencing. These studies have demonstrated reduced ventricular remodeling in myocardial infarction-induced or ischemic models of HF. Preliminary studies using the IL-1 receptor antagonist anakinra post ST-segment elevation myocardial infarction (STEMI) in humans reduced the incidence of subsequent HF,[48,63] demonstrating that a reduction in IL-1β activity decreases ventricular remodeling. A recent trial of an intermediate substrate in the

synthesis of glyburide (16673-34-0) demonstrated inhibition of NLRP3 in a mouse model of acute myocardial infarction.[64]

The main translational focus of immune modulation in HF has been on cytokine proteins; however, clinical trials targeting pro-inflammatory cytokines, such as anti-TNFα therapy, have yet to demonstrate improvements in HF therapy.[24,65] Efforts at immune modulation using disease-modifying agents of rheumatoid diseases (DMARDs), such as anakinra and methotrexate, have demonstrated improvements for chronic inflammatory diseases, such as rheumatoid arthritis, gout and more recently post-infarction HF, but at a cost physically to the patient.[24] Potent anti-inflammatory therapy, anti-TNFα and anti-IL-1β in particular, has a risk for infection that is higher than or equal to the benefit of the therapy.[24] Alternative methods, such as epigenetic regulation of the inflammatory response and targeting upstream components of the signaling cascade have been proposed,[24] however no successful trials in humans have been reported to date.

The NLRP3 inflammasome has been shown to contribute to the development of HF after myocardial infarction.[5] ASC is a key component of the NLRP3 inflammasome and is necessary for caspase-1 mediated activation of IL-1β and IL-18. Inflammation in HF can be regulated by ASC methylation (Figure 1.1). This pathway is important to our understanding of the pathological processes behind myocardial remodeling in HF as our current understanding does not distinguish the individual contributions of the cytokines involved.

Interleukin-1β and Interleukin-18 in Heart Failure

IL-1β is a pyrogenic, pro-inflammatory cytokine that functions as a potent mediator of the inflammatory response and plays a role in cellular activities such as cell proliferation, differentiation, and apoptosis.[46,66] IL-1β induces calcium leakage from the sarcoplasmic reticulum in cardiomyocytes, impairing cardiac contractility.[67,68] IL-1β stimulates nitric oxide production, as evidenced by a corresponding increase in circulating inducible nitric oxide synthase (iNOS), leading to cardiomyocyte apoptosis and tissue remodeling.[65,68] IL-1β is increased in HF, contributes to poor exercise tolerance in persons with systolic HF, and is the key mediator in myocardial remodeling after ischemia-reperfusion injury.[34,68] Modulation of IL-1β attenuates myocardial enlargement and ventricular dysfunction.[69]

IL-18 is a pleiotropic, pro-inflammatory cytokine theorized to play a prognostic and mechanistic role in HF.[8,10] Increased IL-18 levels are positively correlated with New York Heart Association (NYHA) class and are significantly related to HF severity and mortality.[1,8] IL-18 has been shown to increase during acute HF decompensation and remain elevated 90 days after resolution of the acute episode,[8] suggesting a role in myocardial dysfunction. Further, IL-18 is increased in patients with decompensated HF.[10] In the non-failing myocardium, IL-18 is found in precursor form; in contrast, IL-18 in the failing heart is almost completely processed to the active form.[10] IL-18 induces the production of tumor necrosis factor-alpha (TNFα).[8,70] While the inflammatory sequelae of IL-18 in HF have been investigated, modifiable pathways that influence IL-18 production in HF are not well studied.

IL-1β is produced as a precursor protein in response to an inflammatory stimulus.[46,66,71] IL-18 is constitutively expressed as a biologically inactive precursor

molecule that lacks a signal peptide. IL-18 mRNA has a long half-life, thus contributing to steady state production of IL-18. Proform IL-1β and proform IL-18 require caspase-1 dependent proteolytic cleavage for activation.[10,71] In response to a danger signal, caspase-1 is recruited by the adaptor molecule ASC, which leads to inflammasome formation and subsequent IL-1β and IL-18 activation.[10] ASC expression is epigenetically controlled; increased methylation inversely correlates with ASC expression and has been shown to increase with aerobic exercise.[14] Thus, exercise-induced methylation of ASC may prove to be a regulatory pathway of IL-18 expression in HF.

Inducible Nitric Oxide Synthase

Reactive nitrogen species (RNS) are a family of molecules primarily derived from a small, uncharged molecule, NO^-, primarily produced by nitric oxide (NO) synthases, such as inducible nitric oxide synthase (iNOS).[18,37] While physiological levels of RNS are vital in the maintenance of vascular and cardiac cell function, excessive levels lead to cellular toxicity and become highly reactive with other radicals.[18] Excessive RNS levels lead to oxidative stress, an imbalance between free radical generation and detoxification.[18] Oxidative stress is involved in the onset and progression of cardiovascular disease, and increased levels of oxidative stress are found in persons with HF. Oxidative stress decreases cardiac contractility, myocardial Ca^{2+} regulation, and mitochondrial function; the reactive species peroxynitrite ($NO\cdot + \cdot OH- \rightarrow ONOO-$) has been shown to decrease myocardial contractility and disrupt the mitochondrial inner membrane in HF.[18] Aerobic exercise training modulates oxidative stress in animal models of HF,[41] but has not been studied in humans.

Nitric oxide synthases are a family of enzymes that catalyze the production of NO from the amino acid L-arginine.[18] iNOS produces large amounts of NO, which is a key mediator in modulating microcirculatory changes and leukocyte-endothelial interactions.[18,41] Overexpression of iNOS leads to increased NO production and maladaptive ventricular remodeling and is implicated in the pathogenesis of heart failure and myocardial dysfunction.[37,39] While NO may be cardioprotective in some forms, NO specifically produced by iNOS leads to myocardial injury.[65] IL-1β is a powerful inducer if iNOS production.[65] IL-18 has been shown to induce iNOS overexpression and the subsequent release of NO in the inflamed pancreas,[70] however the relationship of IL-18 and iNOS production has not been studied in HF. The aims of this study were to link exercise-induced ASC methylation with decreased circulating IL-1β and IL-18 and subsequently decreased iNOS expression in persons with HF.

Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNFα) is an acute phase pro-inflammatory cytokine implicated in the pathogenesis of heart failure.[9] Animal models have demonstrated that increased circulating TNFα leads to left ventricle dysfunction and cardiomyopathy and worsens heart failure by stimulating cardiomyocyte hypertrophy and induction of myocardial remodeling.[39] TNFα has immediate negative inotropic effects on the myocardium by decreasing intracellular Ca^{2+} release.[34,47] In addition, TNFα directly induces cardiomyocyte hypertrophy and apoptosis.[20,72,73] Increased circulating TNFα is correlated with worsening HF, poor prognosis, and sudden death.[39,72] Exercise trials have

demonstrated that aerobic exercise significantly reduces circulating TNFα in persons with HF.[17,74-77]

Exercise in Heart Failure

Aerobic exercise (AEX) has been shown to be beneficial for most HF patients by altering the deleterious peripheral and central mechanisms, such as inflammatory cytokines, that contribute to HF exacerbations, worsened symptom severity, and poor clinical outcomes.[17,42,74,75,77] In addition, AEX reduces vascular resistance and improves endothelial function as well as the oxidative capacity of peripheral muscles, without a deleterious effect on left ventricular remodeling.[46,74,77] Lower rates of hospitalization, improved physical function and enhanced health-related quality of life (HRQOL) are reported in HF patients who routinely exercise;[17] a meta-analysis of 81 studies also showed trends ($p<0.06$) for lower mortality.[78]

The HF-ACTION trial[15] established the safety and efficacy of moderate-intensity aerobic exercise in patients with stable HF. Participants in the exercise arm completing 4 to 6 metabolic equivalents (MET) hours or more of exercise per week experienced an 18% to 26% reduction in risk, respectively, for the combined endpoint of all-cause mortality and hospitalization.[15,21] A recent meta-analyses also showed significant reductions in brain natriuretic peptide (BNP), a strong prognostic indicator in HF, when weekly energy expenditure exceeded 400 kilocalories (kcals).[79] AEX reduces inflammatory cytokines, including TNF-α, but the effect of aerobic exercise on ASC methylation and IL-18 in persons with HF has not been previously examined.

Peak Oxygen Consumption

Persons with HF often have reduced aerobic capacity and low ventilatory efficiency, leading to poor quality of life.[80] Clinical evaluation of aerobic capacity is measured by peak VO_2, measure of oxygen consumption during effort in a treadmill exercise test, and is a prognostic indicator of decompensation and mortality in HF.[68,80] Cardiac rehabilitation exercise training has been shown to increase peak VO_2; this increase in aerobic capacity is significantly related to decreased BNP and decreased oxidative stress.[81] A two-week treatment with anakinra, an IL-1 receptor antagonist, improved peak VO2 in persons with HF in the absence of an exercise intervention.[68] Thus, aerobic capacity in HF may be related to increased circulating inflammatory cytokines, such as IL-1β and IL-18. AEX-induced ASC methylation may be a non-pharmacologic method of inflammasome modulation leading to decreased inflammation and improved aerobic capacity in HF; however, models of inflammasome modulation related to aerobic capacity have not been reported to date.

Anti-Inflammatory Therapy in Heart Failure

Historically, pharmacologic anti-inflammatory therapies, particularly NSAIDs and the TNFα inhibitors infliximab and etanercept, have either not provided therapeutic benefits or have actually worsened cardiac function in HF.[20,68] TNFα antagonism is thought to have deleterious effects in HF. TNFα confers cytoprotective physiological responses in the heart during acute injury, and loss of that benefit can worsen HF.[82] Additionally, TNFα therapy, such as infliximab, may be cytotoxic to cells due to the drug's effects of fixing complement in cells that express TNFα.[82] Other drugs, such as

interacept, functions to stabilize TNFα, leading to accumulation of increased concentrations of immunoreactive TNFα in peripheral circulation and increasing the duration of TNFα bioactivity.[82] While recent trials with other DMARDs, such as anakinra, have demonstrated prevention of cardiac remodeling post-STEMI,[48,63] the cost-benefit ratio of pharmacologic anti-inflammatory therapy in chronic diseases is worrisome. Additionally, anti-inflammatory drug therapy can lead to compromised host defense or further amplification of inflammatory processes due to the many redundancies.[24] Further exploration of drug therapy that targets the inflammasome is needed. Off-label uses of existing drugs or the development of new drugs may be required. One promising new drug, 16673-34-0 (5-chloro-2-methoxy-N-[2-(4-sulfamoylphenyl)ethyl]benzamide), was found to inhibit cardiomyocyte inflammasome formation post-acute MI in the mouse.[64] Similar drug trials in HF are greatly needed.

Decreased ASC expression through epigenetic control may play an important role in the regulation of inflammation, dampening adverse inflammatory processes without disrupting cellular and tissue homeostasis. The involvement of the inflammasome components in various pathways makes finding specific pharmacologic inflammasome targets a challenge, as it may lead to unintended consequences. For example, by suppressing NLRP3 function, it may be possible also to remove an important intracellular surveyor of danger signals. Some inflammation is necessary for proper healing after insult or injury. The study by Nakajima et al. demonstrated an increase in ASC methylation in older healthy adults after a moderate intensity aerobic exercise program. This study found that ASC methylation is decreased with age and that these age-related changes can be modified with moderate intensity aerobic exercise. While this study did

not examine inflammatory markers related to levels of ASC methylation, regular aerobic exercise may prove to be an effective non-pharmacological modulator of inflammasome activation in HF. In addition, aerobic exercise reduces vascular resistance and improves endothelial function as well as the oxidative capacity of peripheral muscles, without a deleterious effect on ventricular remodeling.[46,74]

Persons with HF often have reduced aerobic capacity and low ventilatory efficiency, leading to worsening symptom severity and poor quality of life.[80] We know that aerobic exercise reduces inflammatory cytokines, including TNF-α, but the effect of aerobic exercise on ASC methylation and IL-18 in persons with HF has not been previously examined. Aerobic exercise-induced ASC methylation may be a non-pharmacologic method of inflammasome modulation leading to decreased inflammation and improved outcomes in HF. We know that aerobic exercise reduces inflammatory cytokines, but the effects of aerobic exercise on ASC methylation, IL-18 and IL-1β have not previously been examined. Further, no studies have been reported to date on changes in DNA methylation in response to exercise in persons with HF. Epigenetic regulation of ASC can be a biological mechanism by which exercise can promote better outcomes in HF. Further research examining mechanisms of change can lead to improved understanding of physiological adaptations and more precise prediction of adverse outcomes in persons with HF.

RESEARCH METHODS AND DESIGN

Research Design

This study was an exploratory sub-study of a 3-year randomized controlled intervention feasibility study. Fifty-four participants were recruited from the Centers of Heart Failure Therapy of Emory University Hospitals. Participants were randomized to receive AEX intervention (N=38) or attention control (N=16) for 3 months. Both groups received two home baseline (BL) visits and bi-monthly phone calls during the maintenance phase, from 3 months to 6 months. Blood draws took place at BL, intervention completion (3 months) and after a 3-month maintenance period (6 months). Outcomes included post-intervention changes in physiological measures (ASC methylation and cytokines) at 3 months and maintenance of changes at 6 months (Table 1.1). Participants were recruited into this sub-study on an opt-in basis by checking a box on the parent study consent form authorizing analysis of DNA methylation (Appendix A).

Table 1.1 *Study Variables, Measures, and Time of Evaluation*

Variable	Measure	Time of Evaluation
Demographics and clinical information	Age, gender, Charlson Comorbidity Index, LVEF, NYHA class, time with HF, etiology of HF, medications	Baseline (BL)
Physical Function	Peak oxygen consumption (Modified Balke)	BL, 3 months (m)
Physiological Status	ASC methylation, IL-1β, IL-18, iNOS	BL, 3m, 6m
Exercise Adherence	Polar HR (intensity duration and frequency), walk calendars, pedometer, nurse telephonic log	3m, 6m

Sample Size

The recruitment goal for the larger feasibility study was 60 participants; therefore, this study was also anticipated to have a sample size of 60. Post-hoc analysis using G*Power software was used to detect a medium-to-large effect size (r^2=0.12) using an estimated sample size of 60, based on multiple linear regression used in Aim 2, which is the most robust aim. However, a sample size of 60 at 80% power can detect large effect sizes in the repeated measures ANOVA (f=0.37 for group main effect, f=0.41 for time main effect and f=0.41 for the group-x-time interaction effect) using an F-Test with a significance level (alpha) of 0.05.

Variables and Measures

Demographics and medical history were collected through chart review and a self-report questionnaire at BL (Table 1.1). Co-morbidities were analyzed using the Charlson comorbidity index (CCI), developed to quantify the risk of death from comorbid diseases. The CCI was developed empirically from 604 patients admitted to a hospital medical service to predict risk of death from comorbid conditions, and compares favorably with the more established Kaplan and Feinstein system.[83]

The plasma cytokines IL-1β, IL-18, and TNFα, were analyzed from plasma that had been separated from collected whole blood and stored at -80°C immediately after collection at the Clinical Research Network (CRN). Plasma cytokines were measured in duplicate using commercially available ELISA kits (eBioscience). Plates were read on a BioTek microplate reader and analyzed using Gen5 software. Curve fitting was selected among linear, quadratic and 4-point based on the best regression coefficient.

DNA for ASC methylation analysis and RNA for pro-IL-18 mRNA, iNOS, and ASC analysis were extracted from the buffy coat that had been separated from collected whole blood and stored at -80°C immediately after collected at the CRN. DNA for ASC methylation was extracted using a commercial kit (PureLink™ Genomic DNA mini kit, Invitrogen), bisulfite treated and sent to EpigenDx for pyrosequencing assays (Table 1.2).

Table 1.2 *Primers and PCR Conditions for ASC Methylation Analysis*

	Forward primers[1]	Reverse primers[2]	Sequencing primers
PCR 1	TGTATTAGTTGGTGTAAG	CACACCCACAACAAC	
	TTTAGAGATAAGTAG	TTCAACTTAA	
PCR conditions			
95 ° C-5 m, (95 ° C-30s; 50 ° C-30 s, 72 ° C-30 s) × 3, (95 ° C-30 s, 58 ° C-30 s, 72 ° C-30 s) x 47, 72 ° C-5 m			
Nested PCR 2	AGGGGATTAAGGGTG	B-CTCCTCCACCAT	GGGATTTTGGAG
		CAAATTCTC	
	TAGTAAGGAA		TTATG
PCR conditions			
95 ° C-5 m, (95 ° C-30s; 58 ° C-30 s, 72 ° C-20 s) × 50, 72 ° C-5 m			

[1]All primers are listed 5' to 3'
[2]B = biotinylated 5' end

mRNA was extracted using a commercial kit (mRNA Catcher™ Plus, Invitrogen). mRNA was converted to cDNA reverse transcriptase PCR (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). IL-18, ASC, and iNOS mRNA were quantified via quantitative real-time PCR (qRT-PCR). GAPDH was used as the reference gene. Primers used for qRT-PCR are listed in Table 1.3. mRNA quantification results were calculated using the ΔΔCT method.[84]

Table 1.3 *Primers for qRT-PCR*

Name	Forward	Reverse
GAPDH	5'-GACCACAGTCCATGCCATCAC-3'	5'-GTCCACCACCCTGTTGCTGTA-3'
ASC	5'-GCCGAGCTCACCGCTAACG-3'	5'-CATCCAGCAGCCACTCAACG-3'
IL-18	5'-CAAGGAAATCGGCCTCTATT-3'	5'-TCCTGGGACACTTCTCTGAA-3'
iNOS	5'-CAAGCCTACCCCTCCAGATG-3'	5'-CATCTCCCGTCAGTTGGTAGGT-3'

Aerobic capacity was assessed using the modified Balke maximal symptom-limited treadmill test[85,86] to determine peak oxygen consumption (peak VO_2). Continuous gas exchange (VMAX Spectra 29 CPET Instrument, Yorba Linda, CA), telemetry, blood pressure, rating of perceived exertion, and oxygen saturation were assessed for each patient 1 minute before , during, and 4 minutes after the exercise test according to American Heart Association guidelines.[80] The test protocol was as follows: 0% incline at 2.0 mph on motorized treadmill for 3 minutes with an increase in incline of 3.5% every 3 minutes for 18 minutes. At 18 minutes, speed increased to 3.0 mph and incline decreased to 12.5%. No participants progressed beyond this point.

Blood Collection Procedures

Baseline, three month and six-month measures were taken at the CRN of the Atlanta Clinical Translational Science Institute by a trained data collector blinded to group assignment. Blood samples were drawn between 7 am and 9 am from patients in a fasting state after quietly resting for 15 minutes according to study protocol by nurses in the CRN. Blood was collected in a vacutainer (10 mL) with EDTA, separated into plasma

and buffy coat, and stored at -80°C in the CRN until processed. The three time points for each participant were analyzed in triplicate.

Intervention description

Both groups received Usual Care (UC) + group assignment (attention control stretching, or moderate intensity AEX) after baseline measures conducted at the CRN. UC includes pharmacologic therapy according to the American College of Cardiology guidelines[86] and patient education and HF self-management. All study patients were given standard printed educational materials (Heart Failure Society of America Modules 1 and 3) regarding HF. Group assignments, schedules and procedures are outlined in Table 1.4. Education, flexibility and stretching provided control for the possible confounding variable of receiving attention from a healthcare professional. Instruction on stretching and flexibility movements was also expected to reduce attrition and patient dissatisfaction and to ensure better concealment of group allocation. These time-equivalent, flexibility/stretching movements served as the placebo exercise condition. In previous pilot evaluations, they were well received but were not strong enough to influence outcomes. The AC education placebo component was delivered as soon as feasible after baseline (T1). The first two weeks of the intervention phase included two home visits to demonstrate stretching and flexibility movements and to discuss educational materials. Weekly telephone calls were then made for the next 10 weeks to discuss educational materials and to answer questions about stretching movements. An appointment was made for participants to come to the CRN to collect T2 measures; this occurred at weeks 13 and 14. From weeks 15-24 bi-monthly telephone contact was used

to encourage continued adherence to the AC intervention. At the end of 24 weeks, a final visit to the CRN was arranged, and T3 measures were taken.

Table 1.4 *Intervention Group Schedules*

Group	T1	1-2 Weeks	3-12 Weeks	13-14 Weeks	15-24 Weeks	24-25 Weeks
UC	Baseline measures	2 home visits for HF education & stretching	Weekly calls	3-month measures	Bi-monthly calls	6-month measures
AEX	Baseline measures	2 home visits for HF education & supervised walk	Weekly calls	3-month measures	Bi-monthly calls	6-month measures

Exercise prescription

The AEX group received the exercise prescription using a progressive, moderate intensity aerobic protocol. To ensure that participants achieve adequate training stimulus, dose-specific exercise was based on maximum heart rate (HR) obtained during symptom-limited, modified Balke treadmill tests[85,86] conducted by an exercise physiologist and supervising cardiologist or nurse practitioner in the CRN. Target heart rate (THR) using the 60% to 70% of maximum HR achieved on the cardiopulmonary exercise test (CPET) was used to monitor the intensity and training response for aerobic exercise. Each participant was provided with an individualized THR zone based on CPET results.[86] Under the supervision of a research nurse, participants began the walking sessions at 60% of THR and increase to 70% by week five as shown in Table 1.5. Pilot studies demonstrated that a progressive, moderate intensity level exercise program yielded positive outcomes with no adverse events, was appealing to participants and resulted in adherence rates of 80% and higher.[87] The first two weeks of the study consisted of two consecutive weekly home visits by the research nurse to demonstrate the use of the HR

monitor and pedometer. The research nurse supervised one walking session during the first two weeks to ensure the safety of the participant and that he/she understood how to use the Polar HR monitor and pedometer and how to complete the walking logs. The weekly schedules are provided in Table 1.5. The time points were chosen based on previous studies that showed the ability to document change in physical function and psychological outcomes at three months; other reports have shown change within 4-12 weeks in this population. The six-month time point was chosen to determine if the changes persisted post exercise intervention, or were diminished.

Table 1.5 *Aerobic Exercise Progression*

	Week 1-2	Week 3-4	Week 5-7	Week 8-9	Week 10-12
Intensity	60%	60%	70%	70%	70%
Duration	30 mins	45 mins	45 mins	45 mins	45 mins
Frequency	3x/wk	3x/wk	3x/wk	3x/wk	3x/wk

Exercise adherence

To be 100% adherent for exercise sessions, participants in the AEX group must have documented walking 3 times each week at the prescribed intensity/duration on the exercise log. Participants recorded exercise sessions on a calendar provided: maximum HR achieved; the rate of perceived exertion (RPE) during walking; and number of steps walked during the walking sessions. Polar HR monitors and pedometers were used to objectively document exercise intensity (i.e., maximum HR and RPE achieved), duration, walking adherence and progression. The Polar HR monitor and pedometer data were downloaded during each home visit during the first two weeks to track exercise duration

and steps walked. At T2, data collected from the HR monitors and pedometers were also downloaded to document exercise adherence. A total summary adherence score was calculated by summing and dividing weekly adherence scores by total number of weeks walked.

Protection of Human Subjects

All study protocols were reviewed and approved by the Institutional Review Board of Emory University (IRB #60752).

Participant Characteristics

All participants in the study met the following criteria. Inclusion criteria included: documented medical diagnosis of NYHA class II or III, aged 40-75 years; left ventricular ejection fraction (LVEF) ≥10% documented within the last year by echocardiogram, cardiac catheterization ventriculography, or radionuclide ventriculography; and receiving medication therapy for HF according to the American College of Cardiology/American Heart Association recommendation guidelines for at least 8 weeks prior to study enrollment. Exclusion criteria included: medical diagnosis of NYHA class I or IV; change in HF therapy within the previous 8 weeks; worsening HF symptoms within the last 5 days; unstable angina; renal insufficiency (serum creatinine > 3.0 mg/dL); fixed rate pacemaker; uncontrolled hypertension, involved in any structured exercise program or exercising 3 or more times per week for a minimum of 30 minutes; hospitalization within the previous 30 days; and any disorder precluding an exercise treadmill test.

Severity of illness was controlled by limiting participants to NYHA class II and III, who are more similar in response to exercise than class I and IV. In addition, severity of illness was controlled by LVEF limitations (LVEF > 10%) and optimal medication therapy for HF. Duration of HF is commonly recorded as NYHA class and LVEF; over time, as therapies become less effective, LVEF decreases and NYHA class increases. Currently, there is little evidence that duration of HF affects outcomes independent of severity, as measured by LVEF and NYHA class.

Rationale for sample characteristics

The age range was selected to avoid confounding effects of age and sarcopenia with outcomes, such as aerobic capacity; participants below the age of 40 years are likely to have HF for other reasons than the majority of the general HF population. Older age (>75) is associated with reduced exercise capacity which may confound the physical function outcome measurement. In addition, persons over the age of 75 are at higher risk for adverse events during exercise testing. Both resting and exercise heart rates are influenced by beta-blockers, which is considered optimal therapy for HF patients with reduced ejection fraction and this was controlled for during analysis. For both exercise testing and training, the heart rate reserve method was used, which takes into account the patient's resting heart rate, thereby reducing the effect of beta-blockade. Best efforts were made to schedule patients for their exercise testing and training a minimum of three hours after taking beta blockers. Patients with an ICD were enrolled if their heart rate limits were set to be higher than the target heart rate for the exercise regimen. Participants with recurrent angina, more severe symptoms, or have uncontrolled hypertension were

excluded due to the higher risk for adverse cardiovascular events during exercise testing and the walking intervention. Because the benefit of exercise is being evaluated, participants who were currently or recently enrolled in an exercise program for the previous eight weeks or were exercising at regular intervals (more than twice per week for 30 minutes) were excluded from the study. Participation in the study was voluntary and participants could withdraw at any time without consequences to themselves, families or communities. The involvement of human subjects in this study involved laboratory blood samples drawn during the same blood draw as the parent study. Therefore, no additional venipuncture occurred for this study.

Sources of Research Materials

Sources of research material included laboratory blood samples obtained at baseline, 3 months, and 6 months. Demographics and medical history taken from the parent study were used for analysis in this study.

Potential Risks

Potential risks were minimal since participants were referred from an academic health sciences center under the care of a cardiologist and receiving optimal medication and device therapies. In addition, excluding patients who are over the age of 75 years, NYHA class IV and have an LVEF < 10 served to reduce the risk of adverse cardiac events. The cardiologist was contacted and agreed that potential participants were eligible for the exercise program. A brief history and physical examination were conducted before aerobic capacity tests (modified Balke) at the ACTSI. If a participant

became fatigued and did not wish to continue, he or she was rescheduled for an additional appointment to complete the remainder of the tests.

The modified Balke, a symptom-limited treadmill test, was administered in the ACTSI by an exercise physiologist and under the sponsorship of the study cardiologist according to the ACC/AHA guidelines.[88] The treadmill test was performed with commercially available equipment (VMAX Spectra 29 CPET Instrument, Yorba Linda, CA) according to the recommendations of the ACC/AHA guidelines.[88] Before the exercise testing, each participant underwent spirometry and remained seated for 2 min to obtain resting oxygen consumption (VO_2). The participant walked for 1 min to warm up to avoid potential muscle soreness. Emergency equipment, medical and nursing staff were immediately available in case the participant had any adverse response. A continuous electrocardiogram and blood pressure readings every one minute were recorded. The participant was continuously monitored during the treadmill test and vital signs taken every one minute for five minutes after the test, then every five minutes. Data from the HF-ACTION study provides evidence of the safety of symptom-limited exercise tests.[89,90] Of the 4,411 symptom-limited exercise tests during five years, no deaths and only two nonfatal, major CV events occurred (0.45 events/1,000 tests). There were also no test-related ICD discharges requiring hospitalization. Based on this review, it was concluded that in NYHA class II-IV patients with severe left ventricular systolic dysfunction, that symptom-limited exercise testing is safe based on no deaths and a rate of nonfatal major CV events that was <0.5 per 1,000 tests.[88]

The potential risks associated with the walking intervention were minimal. Any potential cardiovascular (CV) events that posed a risk to the participant would likely have

been detected during the modified Balke maximal treadmill test. In addition, the duration of walking was limited to 30 minutes during the first 2 weeks and at an intensity level (60%). Participants wore a Polar HR monitor so that HR and intensity level were closely monitored. Participants underwent two supervised walking sessions prior to walking at home unsupervised; the risk associated with the unsupervised session, therefore, was minimal. Participants in the study were provided with detailed instructions on self-monitoring HR, BP, and symptoms associated before, during and after walking. Each participant was provided with a target heart rate range to stay within during the study period. Participants were instructed to wear the Polar HR monitor during each exercise session. The participants were instructed to take their HR, blood pressure (if machine available), and weight prior to and after each walking session and record it in their walking calendar. Participants were instructed to call the research nurse if their blood pressure or HR is outside their normal range. If participants were symptomatic, experience increased shortness of breath or have a greater than two-pound weight gain over the previous 24 hours, they were instructed not to exercise. The participants were instructed to take their medications as usual prior to exercise and instructed on proper attire for exercising. Demonstrations and return demonstrations of the Polar HR monitor ensured they knew how to wear the monitor around the chest as well as troubleshoot when the monitor did not display a HR. They were instructed that if their HR approached within 5-10 beats of the target HR range to slow the walking pace down. They were also instructed if they had an ICD, to keep their HR 15 beats below the firing range at all times (usually set at higher than 170 beats per minute, so it was unlikely to interfere with training stimulus). In addition, they were instructed to monitor their RPE using the Borg

6 to 20 scale, and to keep their RPE at 12-13 during the initial weeks and to progress gradually with instructions to 15 as stipulated in the protocol. Participants in the intervention groups were advised to carry a cell phone when they walked at home in the event of an emergency or sudden event. Specifically, participants were instructed to slow their pace if their HR increased to near THR as previously noted or if they became short of breath. Participants who had ischemic heart disease and prescribed nitroglycerin (NTG) were instructed to carry their NTG with them during each walking sessions. If chest pain were to occur during exercise, the participants were told to stop exercising and to take a NTG as directed by their cardiologist. The participant was also instructed to sit down and to call a relative or friend and to take another NTG if they had chest pain that did not subside. If they were to have chest pain that continued and not relieved by NTG within 10 minutes they were instructed to call 911. If the participant were to become moderately to severely dyspneic where they could not talk while walking, they were instructed to stop walking until the dyspnea subsided. If the dyspnea were to continue once they have stopped walking for several minutes they were instructed to call a relative or friend; if not dissipated they were instructed to go the nearest emergency room. If a participant were to become dizzy while walking, they were instructed to sit down and to get up slowly once the dizziness passes. If an ICD were to fire, the participant was instructed to call 911 if symptoms present or to notify their cardiologist if no symptoms for possible evaluation.

The *potential risk to participants in the AC control stretching/flexibility* movement group was minimal. Participants were taught how to use stretching and flexibility movements by a research nurse.

All patient records were kept in a locked file cabinet in the research office and were accessible only to the PI and the research team. All data was coded by subject identification number, and no identifying information was recorded on the data collection forms. The master list that connected the codes to identifying information will be secured in the research project office. All data maintained in the computerized database (RedCap) was accessible only with a login and protected, encrypted password. After the study is completed, all data will be kept according to regulations in a locked file.

Events

One participant became dizzy while exercising. This study related event was reported to the IRB and determined to be an expected adverse event. One hospitalization and two falls occurred, none of which were study related.

Recruitment and Informed Consent

Participants in the parent study entered into the sub-study on an opt-in basis by selecting a box on the IRB-approved consent form. Participants were recruited through two mechanisms, health provider referral and self-referral, following HIPPA guidelines. Once eligibility was established via phone or in person, the study was fully explained and written informed consent was obtained from each participant by a member of the research team.

Data Analysis

Descriptive statistics were analyzed for all study variables with examination of type and extent of missing data. Data were reviewed for normality assumptions and

outliers, in preparation for analysis. All data were analyzed using SPSS version 23 or SAS version 9.4 (for multilevel modeling analysis using PROC MIXED) and an alpha set at 0.05. For hypothesis testing, data were analyzed according to intention to treat principles. The primary analysis used to test the hypotheses employed a general linear mixed model for repeated measures data. For most variables, the model had one between-subjects variable GROUP with two levels and one within-subjects variable TIME with three levels *(BL, 3 and 6 months*)**.** The test of the hypotheses hinged on the GROUP by TIME interaction as an indication of group differences in the variable of interest over time. A given hypothesis was supported by the finding of a statistically significant GROUP by TIME interaction, *and* the finding that the means are in the hypothesized direction. Multilevel modeling, using PROC MIXED, provided separate estimates of the means for each variable by time and treatment groups. This method allowed to control for attrition. The linear model was fit using restricted maximum likelihood estimation with an appropriate form for the variance-covariance among the repeated measures. Covariates for each hypothesis were selected based on literature documentation of relationships and performance of the data and relationships within this study. The advantages of using PROC MIXED is that the mixed model is capable of handling missing data through the use of restricted maximum likelihood estimation, retaining subjects in the analysis and preserving sample size, and can accommodate covariates as defined in the model. Following the mixed model analysis, Tukey's posthoc comparisons were used to assess change over time in the two groups.

Specific Aim1: Examine the effects of AEX on ASC methylation in persons with heart failure.

H1: Persons with HF, who participate in an AEX intervention, will have increased ASC methylation as compared to attention-control after a 3-month AEX intervention. RQ1: Are intervention effects on ASC methylation sustained 3 months after completing the AEX intervention? Multilevel modeling (PROC MIXED) was used to test group differences in mean changes in ASC methylation from BL to 3 months (H1) and from 3 months to 6 months (RQ1).

Specific Aim2: Examine the relationship of ASC methylation and inflammatory cytokines in persons with HF.

H2: Increased ASC methylation will be related to decreased plasma IL-1β, IL-18, TNFα and with decreased iNOS mRNA expression in persons with HF. Linear regression analysis was used to determine significant relationships between changes in ASC methylation and changes in plasma IL-1β, IL-18, and TNFα and with changes in iNOS mRNA expression.

RQ2a: Does increased ASC methylation, in response to AEX intervention, affect the ratio of proform IL-18 mRNA to plasma IL-18? Linear regression analysis was used to determine significant relationships between ASC methylation and the IL-18 mRNA-plasma IL-18 ratio.

RQ2b: Does increased ASC methylation have a mediating effect on changes in circulating IL-1β and IL-18 in persons with HF? Mediation analysis using PROCESS macro was performed to examine the indirect effects of increased ASC methylation on

the relationship between cytokines at baseline and 3 months. Because the sample size is underpowered to run a full mediation analysis, this research question was designed to be exploratory.

Exploratory Aim: Examine the relationships between levels of ASC methylation, plasma IL-1β, and plasma IL-18 with aerobic capacity, as measured by peak VO_2, in persons with HF. Correlation and multiple linear regression analysis were used to examine significant relationships between levels of ASC methylation, plasma IL-1β and plasma IL-18 with peak VO2 at BL and at 3 months.

Summary

HF is the leading cause of morbidity and mortality in the world, with a prevalence that is projected to increase over time.[91] The economic burden of outpatient and inpatient HF care is increasing in step with the increasing prevalence,[3] heightening the need for effective HF therapies. The role of exercise in HF may prove to be a more important part of HF management than currently realized. Examining molecular targets implicated in the pathological disease processes of worsening HF are vital to our understanding of exercise therapy in HF. Furthermore, uncovering novel molecular pathways in HF that can be modified by aerobic exercise may provide us with therapeutic targets for future HF interventions.

References

1. Eslick GD, Thampan BV, Nalos M, McLean AS, Sluyter R. Circulating interleukin-18 concentrations and a loss-of-function P2x7 polymorphism in heart failure. *Int. J. Cardiol.* 2009;137(1):81-83. doi:10.1016/j.ijcard.2008.05.017
2. van Tassell B, Varma A, Salloum FN, Das A, Seropian IM, Toldo S, et al. Interleukin-1 trap attenuates cardiac remodeling after experimental acute myocardial infarction in mice. *J. Cardiovasc. Pharmacol.* 2010;22:117-122. doi:10.1097/FJC.0b013e3181c87e53
3. Heidenreich PA, Trogdon JG, Khavjou OA, Butler J, Dracup K, Ezekowitz MD, et al. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation.* 2011;123(8):933-944. doi:10.1161/CIR.0b013e31820a55f5
4. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey Jr. DE, Drazner MH, et al. 2013 ACCF/AHA guideline for the management of heart failure. A report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* 2013;62(16):e147-239. doi:doi: 10.1016/j.jacc.2013.05.019
5. Abbate A. The heart on fire: Inflammasome and cardiomyopathy. *Exp. Physiol.* 2013;98(2):385. doi:10.1113/expphysiol.2012.069021
6. Bracey NA, Beck PL, Muruve DA, Hirota SA, Guo J, Jabagi H, et al. The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1β. *Exp. Physiol.* 2013;98(2):462-472. doi:10.1113/expphysiol.2012.068338

7. Paulus WJ, Tschöpe C. A novel paradigm for heart failure with preserved ejection fraction. *J. Am. Coll. Cardiol.* 2013;62(4):263-271.

8. Yamaoka-Tojo M, Tojo T, Inomata T, Machida Y, Osada K, Izumi T. Circulating levels of interleukin 18 reflect etiologies of heart failure: Th1/TH2 cytokine imbalance exaggerates the pathophysiology of advanced heart failure. *J. Card. Fail.* 2002;8(1):21-27.

9. Hedayat M, Mahmoudi MJ, Rose NR, Rezaei N. Proinflammatory cytokines in heart failure: double-edged swords. *Heart Failure Reviews.* 2010;15:543-562.

10. Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, et al. Evidence for altered interleukin (IL)-18 pathway in human heart failure. *The FASEB Journal.* 2004.

11. Naito Y, Tsujino T, Fujioka Y, Ohyanagi M, Okamura H, Iwasaki T. Increased circulating interleukin-18 in patients with congestive heart failure. *Heart & lung : the journal of critical care.* 2002;88:296-297.

12. Okamura H, Tsutsui H, Kashiwamura S-I, Yoshimoto T, Nakanishi K. Interleukin-18: A novel cytokinethat augments both innate and acquired immunity. *Adv. Immunol.* 1998;70:281-312.

13. Lebel-Binay S, Berger A, Zinzindohoué F, Cugnenc P-H, Thiounn N, Fridman WH, et al. Interleukin-18: Biological properties and clinical implications. *Eur. Cytokine Netw.* 2000;11(1):15-26.

14. Nakajima K, Takeoka M, Mori M, Hashimoto S, Sakurai A, Nose H, et al. Exercise effects on methylation of ASC gene. *Int. J. Sports Med.* 2010;31:671-375. doi:10.1055/s-0029-1246140

15. O'Connor CM, Whellan DJ, Lee KL, Keteyian SJ, Cooper LS, Ellis SJ, et al. Efficacy and safety of exercise training in patients with chronic heart failure: HF-ACTION randomized controlled trial. *JAMA.* 2009;301(14):1439-1450.

16. DeMaeyer C, Beckers P, Vrints CJ, Conraads VM. Exercise training in chronic heart failure. *Therapeutic Advances in Chronic Disease.* 2013;4(3):105-117.

17. de Meirelles L, Matsuura C, Resende AD, Salgado ÂA, Pereira NR, Coscarelli PG, et al. Chronic exercise leads to antiaggregant, antioxidant and anti-inflammatory effects in heart failure patients. *European Journal of Preventive Cardiology.* 2013;epub ahead of print.

18. Umar S, van der Laarse A. Nitric oxide and nitric oxide synthase isoforms in the normal, hypertrophic and failing heart. *Mol. Cell. Biochem.* 2010;333:191-201.

19. Tschopp J, Schroder K. NLRP3 inflammasome activation: the convergence of multiple signalling pathwayson ROS production? *Nature Reviews Immunology.* 2010;10:210-215. doi:10.1038/nri2725

20. Chung ES, Packer M, Lo KH, Adedigbo A. Fasanmade, Willerson JT. Randomized, double-blind, placebo-controlled, pilot trial of Infliximab, a chimeric monoclonal antibody to tumor necrosis factor-α, in patients with moderate-to-severe heart failure. Results of the Anti-TNF Therapy Against Cognestive Heart Failure (ATTACH) Trial. *Circulation.* 2003;107:3133-3140.

21. Swank AM, Horton J, Fleg JL, Fonarow GC, Keteyian S, Goldberg L, et al. Modest increase in peak VO2 is related to better clinical outcomes in chronic heart failure patients. Results from Heart Failure and a Controlled Trial to

Investigate Outcomes of Exercise Training. *Circulation Heart Failure.* 2012;5:579-585.

22. Abbate A, Salloum1 FN, Van Tassell B, Vecile E, Toldo S, Seropian I, et al. Alterations in the interleukin-1/interleukin-1 receptor antagonist balance modulate cardiac remodeling following myocardial infarction in the mouse. *PLoS ONE.* 2011;6(11):e27923.

23. Heymans S, Hirsch E, Anker SD, Aukrust P, Balligand J-L, Cohen-Tervaert JW, et al. Inflammation as a therapeutic target in heart failure? A scientific statement from the Translational Research Committee of the Heart Failure Association of the European Society of Cardiology. *European Journal of Heart Failure.* 2009;11:119-129.

24. Tabas I, Glass CK. Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science.* 2013;336:166-172. doi:10.1126/science.1230720

25. Horsburgh S, Robson-Ansley P, Adams R, Smith C. Exercise and inflammation-related epigenetic modifications: focus on DNA methylation. *Exerc. Immunol. Rev.* 2015;21:26-41.

26. Packer M. The neurohormonal hypothesis: a theory to explain the mechanism of disease progression in heart failure. *J. Am. Coll. Cardiol.* 1992;20(1):248-254.

27. Cohn JN, Archibald DG, Ziesche S, Franciosa JA, Harston E, Tristani FE, et al. Effect of vasodilatory therapy on mortality in chronic congestive heart failure: results of a Veterans Administration cooperative study. *N. Engl. J. Med.* 1986;314:1547-1522. doi:10.1056/NEJM198606123142404

28. Francione JA, Jordan RA, Wilen MM, Leddy CL. Minoxidil in patients with left heart failure: contrasting heodynamic and clinical effects in a controlled trial. *Circulation.* 1984;63:652-657.

29. Packer M, Carver JR, Rodeheffer RJ, Ivanhoe RJ, DiBianco R, Zeldis SM, et al. Effect of oral milrinone on mortality in severe chronic heart failure. *N. Engl. J. Med.* 1991;325:1468-1475. doi:10.1056/NEJM199111213252103

30. Seta Y, Shan K, Bozkurt B, Oral H, Mann DL. Basic mechanisms in heart failure: the cytokine hypothesis. *J. Card. Fail.* 1996;2(3):243-249.

31. Hofmann U, Frantz S. How can we cure a heart ‘‘in flame’’? A translational view on inflammation in heart failure. *Basic Res. Cardiol.* 2013;108:356-375. doi:10.1007/s00395-013-0356-y

32. El-Menyar AA. Cytokines and myocardial dysfunction: state of the art. *J. Card. Fail.* 2008;14(1):61-74. doi:10.1016/j.cardfail.2007.09.006

33. von Haehling S, Schefold JC, Lainscak M, Doehner W, Anker SD. Inflammatory biomarkers in heart failure revisited: Much more than innocent bystanders. *Heart Failure Clinician.* 2009;5:549-560. doi:10.1016/j.hfc.2009.04.001

34. Kawaguchi M, Takahashi M, Hata T, Kashima Y, Usui F, Morimoto J, et al. Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation.* 2011;123:594-604. doi:10.1161/CIRCULATIONAHA.110.982777

35. Mezzaroma E, Toldoa S, Farkasb D, Seropiana IM, Tassellb BWV, Sallouma FN, et al. The inflammasome promotes adverse cardiac remodeling following acute

myocardial infarction in the mouse. *PNAS.* 2011;108(49):19725-19730. doi:10.1073/pnas.1108586108

36. Nakayama H, Otsu K. Translation of hemodynamic stress to sterile inflammation in the heart. *Trends in Endocrinology and Metabolism.* 2013;24(11):546-553. doi:10.1016/j.tem.2013.06.004

37. Otani H. The role of nitric oxide in myocardial repair and remodeling. *Antioxidant & Redox Signaling.* 2009;11(8):1913-1928. doi:10.1089/ARS.2009.2453

38. Vaduganathan M, Greene SJ, Butler J, Sabbah HN, Shantsila E, Lip GYH, et al. The immunological axis in heart failure: importance of the leukocyte differential. *Heart Failure Rev* 2013;18(6):835-845. doi:10.1007/s10741-012-9352-9

39. Elahi M, Asopa S, Matata B. NO-cGMP and TNF-α counter regulatory system in blood: Understanding the mechanisms leading to myocardial dysfunction and failure. *Biochim. Biophys. Acta.* 2010;1772:5-14.

40. Wenceslau CF, McCarthy CG, Szasz T, Spitler K, Goulopoulou S, Webb RC. Mitochondrial damage-associated molecular patterns and vascular function. *Eur. Heart J.* May 2014;35(18):1172-1177. doi:10.1093/eurheartj/ehu047

41. Campos JC, Gomes KMS, Ferreira JCB. Impact of exercise training on redox signaling in cardiovascular diseases. *Food Chem. Toxicol.* 2013;62:107-119. doi:10.1016/j.fct.2013.08.035

42. Vaduganathan M, Greene SJ, Butler J, Sabbah HN, Shantsila E, Lip GYH, et al. The immunological axis in heart failure: importance of the leukocyte differential. *Heart Failure Reviews.* 2012;Epub ahead of print.

43. Glezeva N, Baugh JA. Role of inflammation in the pathogenesis of heart failure with preserved ejection fraction and its potential as a therapeutic target. *Heart Failure Reviews.* 2014;19(5):681-694. doi:10.1007/s10741-013-9405-8

44. Schroder K, Tschopp J. The inflammasome. *Cell Adhes. Commun.* 2010;140(821-832). doi:10.1016/j.cell.2010.01.040

45. Rathinam VAK, Vanaja SK, Fitzgerald KA. Regulation of inflammasome signaling. *Nature Immunology.* 2012;13(4):333-342.

46. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 2009;27:519-550.

47. Schroder K, Zhou R, Tschopp J. The NLRP3 inflammasome: A sensor for metabolic danger? *Science.* 2010;327:296-300. doi:10.1126/science.1184003

48. Abbate A, Kontos MC, Grizzard JD, Biondi-Zoccai GGL, Van Tassell B, Robati R, et al. Interleukin-1 blockade with Anakinra to prevent adverse cardiac remodeling after acute myocardial infarction (Virginia Commonwealth University Anakinra Remodeling Trial [VCU-ART] pilot study). *Am. J. Cardiol.* 2010;105:1371-1377. doi:10.1016/j.amjcard.2009.12.059

49. Takahashi M. NLRP3 inflammasome as a novel player in myocardial infarction. *International Heart Journal.* 2014;55:101-105.

50. Taniguchi S, Sagara J. Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC. *Seminars in Immunopathology.* 2007;29:231-238.

51. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. *Cell.* 2014;157:1013-1022. doi:10.1016/j.cell.2014.04.007

52. Fedak PWM, Verma S, Weisel RD, Li R-K. Cardiac remodeling and failure. From molecules to Man (part 1). *Cardiovascular Pathology.* 2005;14:1-11. doi:10.1016/j.carpath.2001.12.002

53. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. *Nat. Biotechnol.* 2012;481:278-286. doi:10.1038/nature10759

54. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, et al. Cutting edge: NF-κB activating pattern recoginition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* 2009;183:787-791. doi:10.4049/jimmunol.901363

55. Sutterwala FS, Haasken S, Cassel SL. Mechanism of NLRP3 inflammasome activation. *Ann. N. Y. Acad. Sci.* 2014. doi:10.1111/nyas.12458

56. Martinon F, Mayor A, Tschopp J. The inflammasomes: Guardians of the body. *Annu. Rev. Immunol.* 2009;27:229-265. doi:10.1146/annurev.immunol.021905.132715

57. Kanneganti TD, M L, Nunez G. Intracellular NOD-like receptors in host defense and disease. *Immunity.* 2007;27:549-559.

58. Lamkanfi M, Dixit VM. Inflammasomes and their roles in health and disease. *Annu. Rev. Cell Dev. Biol.* 2012;28:137-161. doi:10.1146/annurev-cellbio-101011-155745

59. Bracey NA, Gershkovich B, Chun J, Vilaysane A, Meijndert HC, James R. Wright J, et al. Mitochondrial NLRP3 induces reactive oxygen species to promote Smad signaling and fibrosis independent from the inflammasome. *J. Biol. Chem.* 2014. doi:0.1074/jbc.M114.550624

60. Wilson SP, Cassel SL. Inflammasome-mediated autoinflammatory disorders. *Postgrad. Med. J.* 2010;122(5):125-133. doi:10.3810/pgm.2010.09.2209

61. Salminen A, Kauppinen A, Hiltunen M, Kaarniranta K. Epigenetic regulation of ASC/TMS1 expression: potential role in apoptosis and inflammasome function. *Cell. Mol. Life Sci.* 2014;71:1855-1864. doi:10.1007/s00018-013-1524-9

62. Butts B, Gary RA, Dunbar SB, Butler J. Methylation of Apoptosis-Associated Speck-Like Protein With a Caspase Recruitment Domain and Outcomes in Heart Failure. *J. Card. Fail.* Dec 14 2015. doi:10.1016/j.cardfail.2015.12.004

63. Abbate A, Van Tassell BW, Biondi-Zoccai G, Kontos MC, Grizzard JD, Spillman DW, et al. Effects of interleukin-1 blockade with anakinra on adverse cardiac remodeling and heart failure after acute myocardial infarction [from the Virginia Commonwealth University-Anakinra Remodeling Trial (2) (VCU-ART2) pilot study]. *Am. J. Cardiol.* 2013;111(10):1394-1400. doi:10.1016/j.amjcard.2013.01.287

64. Marchetti C, Chojnacki J, Toldo S, Mezzaroma E, Tranchida N, Rose SW, et al. A novel pharmacologic inhibitor of the NLRP3 inflammasome limits myocardial injury after ischemia-reperfusion in the mouse. *J. Cardiovasc. Pharmacol.* 2014;63:316-322. doi:10.1097/FJC.0000000000000053

65. Pomerantz BJ, Reznikov LL, Harken AH, Dinarello CA. Inhibition of caspase 1 reduces human myocardial ischemic dysfunction via inhibition of IL-18 and IL-1β. *Proc. Natl. Acad. Sci. U. S. A.* 2001;98(5):2871-2876.

66. Dinarello CA. A clinical perspective of IL-1b as the gatekeeper of inflammation. *Eur. J. Immunol.* 2011;41:1203-1217.

67. Van Tassell B, Seropian IM, Toldo S, Mezzaroma E, Abbate A. Interleukin-1b induces a reversible cardiomyopathy in the mouse. *Inflamm. Res.* 2013;62:637-640.

68. van Tassell B, Arena RA, Toldo S, Mezzaroma E, Azam T, Seropian IM, et al. Enhanced interleukin-1 activity contributes to exercise intolerance in patients with systolic heart failure. *PLoS ONE.* 2012;7(3):e33438. doi:10.1371/journal.pone.0033438

69. Abbate A, Van Tassell B, Seropian IM, Toldo S, Robati R, Varma A, et al. Interleukin-1b modulation using a genetically engineered antibody prevents adverse cardiac remodelling following acute myocardial infarction in the mouse. *European Journal of Heart Failure.* 2010;12:319-322.

70. Ueno N, Kashiwamura S-i, Ueda H, Okamura H, Tsuji NM, Hosohara K, et al. Role of interleukin 18 in nitric oxide production and pancreatic damage during acute pancreatitis. *Shock.* 2005;24(6):564-570.

71. Dinarello CA, Simon A, van der Meer J. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nature Reviews Drug Discovery.* 2012;11:633-652.

72. Bozkurt B, Torre-Amione G, Warren MS, Whitmore J, Soran OZ, Feldman AM, et al. Results of targeted antitumor necrosis factor therapy with etanercept (ENBREL) in patients with advanced heart failure. *Circulation.* 2001;103:1044-1047.

73. Burkard T, Pfister O, Rickli H, Follath F, Hack D, Zaker R, et al. Prognostic impact of systemic inflammatory diseases in elderly patients with congestive heart failure. *QJM.* 2013;Epub ahead of print.

74. Feiereisen P, Vaillant M, Gilson G, Delagardelle C. Effects of different training modalities on circulating anabolic/catabolic markers in chronic heart failure. *Journal of Cardiopulmonary Rehabilitation and Prevention.* 2013;33:303-308.

75. Smart NA, Steele M. The effect of physical training on systemic proinflammatory cytokine expression in heart failure patients: a systematic review. *Congestive Heart Failure.* 2011;17(3):110-114.

76. Tsarouhas K, Tsitsimpikou C, Haliassos A, Georgoulias P, Koutsioras I, Kouretas D, et al. Study of insulin resistance, TNF-α, total antioxidant capacity and lipid profile in patients with chronic heart failure under exercise. *In Vivo.* 2011;25(6):1031-1037.

77. Nunes RB, Alves JP, Kessler LP, Lago PD. Aerobic exercsie improves the inflammatory profile correlated with cardiac remodeling and function in chronic heart failure rats. *Clin. Chest Med.* 2013;68(6):876-882.

78. ExTraMATCH Collaborative. Exercise training meta-analysis of trials in patients with chronic heart failure (ExTraMatch). *BMJ.* 2004;328(7433). doi:10.1136/bmj.37938.645220.EE

79. Smart NA, Meyer T, Butterfield JA, Faddy SC, Passino C, Malfatto G, et al. Individual patient meta-analysis of exercise training effects on systemic brain natriuretic peptide expression in heart failure. *European Journal of Preventive Cardiology.* 2012;19(3):428-435.

80. Balady GJ, Arena R, Sietsema K, Myers J, Coke L, Fletcher GF, et al. Clinician's Guide to cardiopulmonary exercise testing in adults: a scientific statement from the American Heart Association. *Circulation.* 2010;122:191-225.

81. Fukuda T, Kurano M, Fukumura K, Yasuda T, Iida H, Morita T, et al. Cardiac rehabilitation increases exercise capacity with a reduction of oxidative stress. *Korean Circulation Journal.* 2013;43:481-487.

82. Mann DL. Recent insights into the role of tumor necrosis factor in the failing heart. *Heart failure reviews.* 2001;6:71-80.

83. Charlson M, Pompei P, Ales K, MacKenzie C. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J. Chronic Dis.* 1987;40(5):373-383.

84. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* Dec 2001;25(4):402-408. doi:10.1006/meth.2001.1262

85. Balke B, Ware RW. An experimental study of fitness of Air Force personnel. *U.S. Armed Forces Medical Journal.* 1959;10:678-688.

86. Gibbons RJ, Balady GJ, Bricker JT, Chaitman BR, Fletcher GF, Froelicher VF, et al. ACC/AHA 2002 guideline update for exercise testing: summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1997 Exercise Testing Guidelines). *Circulation.* 2002;106(14):1883-1892.

87. Najafi F, Jamrozik K, Dobson AJ. Understanding the 'epidemic of heart failure': a systematic review of trends in determinants of heart failure. *European Journal of Heart Failure.* 2009;11(5):472-479.

88. Keteyian SJ, Isaac D, Thadani U, Roy BA, Bensimhon DR, McKelvie R, et al. Safety of symptom-limited cardiopulmonary exercise testing in patients with chronic heart failure due to severe left ventricular systolic dysfunction. *Am. Heart J.* 2009;158(4 Suppl):S72-S77.

89. Piccini JP, Hellkamp AS, Whellan DJ, Ellis SJ, Keteyian SJ, Kraus WE, et al. Exercise training and implantable cardioverter-defibrillator shocks in patients with heart failure: results from HF-ACTION (Heart Failure and A Controlled Trial Investigating Outcomes of Exercise TraiNing). *JACC. Heart failure.* Apr 2013;1(2):142-148. doi:10.1016/j.jchf.2013.01.005

90. O'Connor CM, Whellan DJ, Lee KL, Keteyian SJ, Cooper LS, Ellis SJ, et al. Efficacy and safety of exercise training in patients with chronic heart failure: HF-ACTION randomized controlled trial. *JAMA.* Apr 8 2009;301(14):1439-1450. doi:10.1001/jama.2009.454

91. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics - 2013 update: A report from the American Heart Association. *Circulation.* 2013;127:e6-e245.

Chapter II

The Importance of NLRP3 Inflammasome in Heart Failure

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Abstract

Patients with heart failure continue to suffer adverse health consequences despite advances in therapies over the last two decades. Identification of novel therapeutic targets that may attenuate disease progression is therefore needed. The inflammasome may play a central role in modulating chronic inflammation and in turn affecting heart failure progression. The inflammasome is a complex of intracellular interaction proteins that trigger maturation of pro-inflammatory cytokines interleukin-1beta and interleukin-18 to initiate the inflammatory response. This response is amplified through production of tumor necrosis factor α and activation of inducible nitric oxide synthase. The purpose of this review is to discuss recent evidence implicating this inflammatory pathway in the pathophysiology of heart failure.

The syndrome of heart failure (HF) results from various structural or functional impairments in cardiac function leading to an inability to maintain cardiac output at normal filling pressures.[1,2] HF remains a major cause of morbidity and mortality in the United States and is the leading cause of hospitalization among individuals over age 65, leading to costs of care exceeding 31 billion dollars annually.[3] Over the past two decades, advances in pharmacological and device therapies for HF have significantly improved prognosis for HF patients with low ejection fraction, however, the overall prognosis continues to be poor for these patients with mortality rates approaching 50% in 5 years.[4] Therefore, attenuating HF disease progression remains an important goal. Identification of novel pathways and effectively intervening on potential therapeutic targets may slow HF disease progression. It is known that HF is associated with a low-grade chronic inflammation leading to adverse cardiac remodeling.[5] In this review, we discuss advances and recent evidence regarding the inflammatory pathway in the pathophysiology of HF.

Importance of Inflammation in Heart Failure

Studies with ACE inhibitors, beta-blockers, and aldosterone antagonists all showed benefit in HF patients with low ejection fraction.[6] However, the persistent high risk for mortality among these patients suggest that neurohormonal activation does not fully explain HF progression. Inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin 1 (IL-1) and 6 (IL-6), and C-reactive protein (CRP) are all increased in HF and their levels are related to HF severity and prognosis.[7] These cytokines are thought to modulate myocardial remodeling, myocyte hypertrophy and

apoptosis, decreased contractility, increased fibrosis, and other adverse structural changes.[8-10] These findings have led to the "cytokine hypothesis" of HF progression.[7,10] Originally it was felt that inflammatory cytokines in HF represents an epiphenomenon, however, recent evidence is suggestive of its mechanistic role.[11] Initial HF studies focused on individual cytokines, however, uncovering pathophysiological processes of myocardial remodeling requires further study of the inflammatory pathways and the underlying mechanisms of cytokine activation.

Danger-associated molecular patterns (DAMPs)

Sterile inflammation in HF is initiated by danger-associated molecular patterns (DAMPs), which are host-derived molecules indicative of cellular damage and has been shown to modulate irreversible myocardial changes, such as fibrosis, apoptosis and hypertrophy.[12-14] Proposed mechanisms of DAMP formation in HF include mitochondrial dysfunction, cellular death, ischemia, cardiac load and oxidative stress.[15-19] Mitochondrial dysfunction and necrotic or apoptotic cardiomyocyte death lead to the release of cellular components such as nuclear and mitochondrial nucleic acids, extracellular ATP, protein aggregates, and other debris.[15,16] Transient ischemia and reperfusion injury, myocardial under perfusion, and other sources of oxidative stress lead to the production of reactive oxygen and nitrogen products, which are powerful DAMPs associated with ventricular remodeling.[1,20] Increased ventricular filling pressures, cavity distension, congestion, shear stress, and other alterations in loading leads to myocardial injury. Byproducts of this injury are detected by myocytes and immune cells as DAMPs and lead to an accelerated sterile inflammation in HF.[17,19] The inflammatory response

amplifies the production of DAMPs, resulting in a positive-feedback loop accelerating HF pathophysiology.[16] Increased cardiac pressure and poor pump function directly trigger activation of inflammatory cells, such as peripheral monocytes, which aggregate in the heart and are released into circulation.[18,19] Activated inflammatory cells release pro-inflammatory cytokines, such as TNFα, which magnify the inflammatory process and contribute to fibrotic changes in the myocardium and progressive remodeling.[5,14,19,21,22]

NLRP3 Inflammasome

DAMP-activated inflammation occurs via the NLRP3 inflammasome, a complex of intracellular interaction proteins that recognize DAMPs and triggers maturation of pro-inflammatory cytokines to initiate and amplify the inflammatory response.[23-25] The inflammasome is composed of a NOD (nucleotide binding oligomerization domain)-like receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and pro-caspase-1 (Figure 1).[26-28] The activated inflammasome cleaves pro-caspase-1 into the active enzyme caspase-1.[29] Caspase-1 in turn activates IL-1 family proinflammatory cytokines IL-1β and IL-18, by cleavage of pro-IL-1β and pro-IL-18 into active forms.[24,30-32] Thus, the inflammasome is a powerful mediator of the immune response via caspase-1 activation of IL-1β and IL-18. The NLRP3 inflammasome can also induce pyroptosis in a caspase-1-dependent manner.[27] Loss of cardiomyocytes via pyroptosis reduces contractile reserve leading to HF progression.[33] In addition, as cytosolic components are released with pyroptosis, extracellular ASC becomes a danger signal and functions to initiate further inflammasome formation. ASC in extracellular space continues to activate caspase-1, propagating the inflammatory cascade.[33]

Inflammasome Activation: Both exogenous (pathogens, toxins) and endogenous (DAMPs) molecules activate NLRP3 inflammasome.[28,34,35] Inflammasome formation and activation requires two signals: a priming and an activating signal.[36] First, danger signals (DAMPs) activate the transcription factor NF-κB, leading to the production of NLRP3 and pro-IL-1β (Figure 1).[34] Other components of the NLRP3 inflammasome pathway (ASC, pro-caspase-1, and pro-IL-18) are readily available in steady state.[29] While the definitive mechanism of NLRP3 inflammasome activation has yet to be uncovered, proposed mechanisms include cation movement (such as K^+ efflux or $Ca2^+$ influx), mitochondrial membrane dysfunction, frustrated phagocytosis, production of reactive oxygen species, and direct binding of oxidized mitochondrial DNA to NLRP3 itself.[28,29,36,37] This two-step process likely represents a regulatory checkpoint in inflammasome activation. Assembly of the inflammasome occurs when the N-terminal pyrin domain (PYD) of NLRP3 interacts with the PYD of ASC in a homotypic fashion, leading to recruitment and activation of caspase-1 (Figure 1).[14,22,23,36] Further work is needed to elucidate mechanisms of inflammasome activation in HF.

NLRP3: NLR family, pyrin domain-containing 3 (NLRP3, aka NALP3 or cryopyrin) is a pattern recognition receptor (PRR) implicated in the pathogenesis of inflammation in chronic disease.[35] NLRP3 transcription is regulated by nuclear factor κB (NF-κB) in the presence of a danger signal, e.g. DAMPs. NLRP3 is made up of 3 functional domains: C-terminal leucine-rich repeats (LRR) with regulatory function, a NACHT domain that has ATPase activity, and PYD that serves as a death-fold domain.[22,30,35] NLRs are believed

to play a role in the cytosol analogous to that of the toll-like receptors (TLRs) in the plasma membrane. Like other NLR family receptors, NLRP3 functions to guard the intracellular environment to maintain homeostasis.[27] NLRP3 differs from other nod-like receptors in that physiological expression levels are not sufficient for inflammasome activation. NLRP3 expression is up regulated via NF-κB upon the sensing of a danger signal. NLRP3 functions as both an intracellular PRR, surveying the cytosol for danger signals, and as a platform protein in inflammasome formation, initiating an inflammatory response.[27] NLRP3 is expressed in a number of cells, e.g. leukocytes, myocytes, and cardiac fibroblasts.[38] NLRP3 knockout mice have smaller areas of infarct in an experimental model of acute myocardial infarction;[13] constitutively active NLRP3 leads to uncontrolled activation of the inflammasome.[39] There is no evidence of transcriptional control of NLRP3 to date.

ASC: ASC is a vital component of the inflammasome and functions to recruit caspase-1 to the inflammasome complex.[5,12,31,40] ASC is necessary for activation of pro-caspase-1 into caspase-1, which in turn is necessary for activation of IL-1 family cytokines, such as IL-1β and IL-18.[5,12] ASC deficiency lessens inflammatory response to ischemia-reperfusion injury in the myocardium.[14] ASC plays a vital role in the activation of the inflammasome and in the inflammatory response to danger signals.

Caspase-1: Caspase-1 is a cysteine protease that function as an effector protein in NLRP3 inflammasome. Caspase-1 is produced by the cell as a zymogen, pro-caspase-1, which undergoes autocatalysis for activation upon a homotypic interaction with the

CARD domain on ASC.[37,40] Activated caspase-1 is involved in the recruitment of innate immune cells to sites of inflammation, induces pyroptosis, and is the primary activator of IL-1β and IL-18.

Interleukin-1β and Interleukin-18: IL-1β is a pro-inflammatory cytokine and plays a role in cellular activities such as cell proliferation, differentiation and apoptosis [41,42]. IL-1β induces calcium leakage from the sarcoplasmic reticulum in myocytes, impairing cardiac contractility [43,44]. IL-1β stimulates nitric oxide production, as evidenced by a corresponding increase in circulating inducible nitric oxide synthase (iNOS), leading to cardiomyocyte apoptosis and tissue remodeling [44,45]. IL-1β is increased in HF and is associated with poor exercise tolerance and with remodeling after ischemia-reperfusion injury [14,44]. Modulation of IL-1β attenuates myocardial enlargement and ventricular dysfunction [46].

IL-1β is produced as a precursor protein in response to an inflammatory stimulus, while IL-18 is constitutively expressed as a biologically inactive precursor molecule lacking a signal peptide. [41,42,47] Both proform IL-1β and proform IL-18 require caspase-1 dependent proteolytic cleavage for activation.[47,48] Increased IL-18 levels are correlated with functional class and mortality in HF.[48] IL-18 is increased during acute HF and remains elevated after discharge. [1,49] In the non-failing myocardium, IL-18 is found in precursor form; in contrast, in the failing heart it is almost completely processed to active form. [48] IL-18 induces the production of TNFα. [49,50] While the inflammatory squelae of

IL-18 in have been investigated; modifiable pathways that increase IL-18 production in HF are not well studied.

Inducible Nitric Oxide Synthase: Reactive nitrogen species (RNS) are molecules derived from a small uncharged molecule NO^-, primarily produced by nitric oxide (NO) synthases, such as iNOS.[17,51] While physiological levels of RNS are vital in the maintenance of vascular and cardiac cell function, excessive levels lead to toxicity and become highly reactive with other radicals.[51] Excessive RNS levels lead to oxidative stress, an imbalance of free radical generation and detoxification.[51] Oxidative stress is involved in the onset and progression of HF. Oxidative stress decreases cardiac contractility, myocardial Ca^{2+} regulation and mitochondrial function; the reactive species peroxynitrite has been shown to decrease myocardial contractility and disrupt the mitochondrial inner membrane in HF.[51] Nitric oxide synthases are enzymes that catalyze the production of NO.[51] iNOS produces NO, which is a key mediator in modulating microcirculatory changes and leukocyte-endothelial interactions [20,51] Over expression of implicated in the pathogenesis of HF.[17,18] While NO may be cardioprotective in some forms, NO specifically produced by iNOS leads to myocardial injury.[45] IL-1β is a powerful inducer of iNOS production.[45] IL-18 has been shown to induce iNOS overexpression and the subsequent release of NO in the inflamed pancreas,[50] however the relationship of IL-18 and iNOS production has not been studied in HF. The proposed NLRP3 inflammasome pathway links ASC methylation with decreased circulating IL-1β and IL-18 and subsequent decreased iNOS mRNA expression in persons with HF.

Tumor necrosis factor-alpha: TNFα is a pro-inflammatory cytokine that is not expressed in healthy myocardium. [52] Animal models have demonstrated that increased circulating TNFα stimulate myocyte hypertrophy and remodeling.[18] TNFα has negative inotropic effects by decreasing intracellular Ca^{2+} release.[14,22] In addition, TNFα directly induces cardiomyocyte hypertrophy and apoptosis.[8,53,54] Increased circulating TNFα is correlated with worsening HF, poor prognosis and sudden death.[18,53]

NLRP3 Inflammasome in Cardiac Remodeling

The work examining the inflammasome in cardiac remodeling and repair has primarily focused on fibroblasts, which comprise up to two-thirds of cells in cardiac tissue.[14] The NLRP3 inflammasome in the myocardial fibroblast is the initial sensor of DAMPs after myocardial injury, and thus is primarily responsible for the initiation of the inflammatory response in injured cardiac tissue. Fibroblasts have been shown to activate the inflammasome via reactive oxygen species and K+ efflux after myocardial ischemic injury.[14,55]

Fibroblasts are able to withstand oxidative stress and thus are able to respond to hypoxia and initiate a hypoxic immune response. In the face of hypoxia, fibroblasts develop an inflammatory and fibrogeneic phenotype, leading to increased cytokines, inflammatory cell infiltration, myofibroblast transdifferentiation, and increased collagen production.[14,55] Ischemic insult leads to ROS and K+ efflux, which activates the inflammasome in the fibroblast. The activated inflammasome produces IL-1β, which activates the initial immune response. The inflammatory fibroblast releases chemokines that recruit macrophages and neutrophils to the site of insult. These recruited leukocytes

also contain inflammasomes that release IL-1β and IL-18, further propagating the inflammatory response after ischemic insult or injury. Thus, the cardiac fibroblasts play a central role in the initiation and enhancement of myocardial injury after ischemia.

NLRP3 plays a role in cardiac fibroblast differentiation through the NACHT domain, independent of inflammasome formation. In cardiac fibroblasts, NLRP3 was found to localize to mitochondria as a regulator of mitochondrial ROS (mROS) production and augment R-Smad signaling, ultimately leading to profibrotic gene expression.[56]

Using a calcineurin transgene mouse model of inflammatory and hypertrophic cardiomyopathy, Bracey et al.[12] demonstrated that the inflammasome, via IL-1β activation, plays a role in pyroptosis during the development of HF and has direct effects on Ca2+ homeostasis, myocardial contractility, and excitation-contraction coupling. NLRP3 can also play a role in myocardial death in an inflammasome-independent manner; in cardiomyocytes, the NLRP3 inflammasome can lead to loss of myocardium by pyroptosis, independent of IL-1β.[13]

In a sample of patients with acute myocarditis, more inflammasome-containing leukocytes were found in myocardial tissue of persons with reduced EF (<40%) and those with more severe HF, as categorized by NYHA Class III and IV, than others.[57] While this is merely associative, and does not demonstrate causality, the continued presence of increased inflammasome formation in injured and stressed myocardium suggests a mechanistic role in the pathogenesis and worsening of HF. Although the NRLP3 inflammasome is implicated in the development of HF, the mechanisms of adverse myocardial remodeling are not completely understood. Further work to elucidate the

mechanisms of myocardial remodeling and repair in the pathogenesis of and worsening of HF are warranted.

Mechanisms of Myocardial Epigenetic Regulation

The primary epigenetic mechanisms linked to the pathogenesis of HF are chromatin modifications, histone modifications, DNA methylation, and microRNAs (miRNA). These epigenetic mechanisms control key processes such as cardiomyocyte hypertrophy, fibrosis and cardiac failure. Several studies have focused on genome-wide mapping of global epigenetic differences between HF and healthy controls,[58-60] which have led to the discovery of several mechanisms and pathways of myocardial epigenetic regulation in HF. Epigenetic changes in promoter regions of p53 response elements, such as complete demethylation of p21 or complete methylation of cyclin D1, are related to cell cycle arrest and have been implicated in the development of diabetic cardiomyopathy.[61] Altered Dicer expression modulates cardiac function and cardiac electrophysiology through disturbed ion channel function, presumably through altered miRNA expression.[61] Several genes related to angiogenesis, including PCAM1, are differentially methylated in HF, suggesting epigenetic regulation of the angiogenic process during HF pathogenesis.[62] Changes in histone deacetylases, such as loss of HDAC1 and HDAC2 in cardiac tissue, lead to in arrhythmias and HF.[63]

Pathogenic cardiac remodeling leading to HF is characterized by a re-activation of fetal programming genes and a repression of adult genes.[64] At the onset of HF, reprogramming of gene expression leads to changes in gene expression, including downregulation of α-MHC (major histocompatibility complex) and SERCA (sarcoplasmic reticulum Ca2+ ATPase) genes and reactivation of the fetal cardiac genes,

ANF (atrial natriuretic factor) and BNP (B-type natriuretic peptide). This reprogramming causes structural and electrophysiological changes that lead to HF.[65] In a model of pressure overload-induced HF, histone acetylation and chromatin modifications led to transcriptional reprogramming and fetal gene expression. These modifications resulted in the de novo synthesis of contractile and structural proteins, and ultimately cardiac hypertrophy.[64]

While epigenetic regulation of the myocardial changes implicated in the pathogenesis of HF have yet to be linked to inflammasome formation, increased circulating IL-18 had been shown to lead to epigenetic changes in the chromatin of ANF and MyHC (myosin heavy chain) genes. These chromatin changes are associated with cardiac hypertrophy via upregulation of β-MyHC and down regulation of α-MyHC, indicative of fetal reprogramming.[66,67] Expression of a vital regulatory component of the NLRP3 inflammasome, ASC, is controlled through epigenetic modification via DNA methylation. ASC methylation is inversely correlated with ASC protein expression and is silenced by overexpression of DNA methyltransferase.[36,37] Hypermethylation leads to an inactive state in which no ASC protein is expressed, while complete demethylation induces apoptosis via p53 and TRAK activation.[36] Thus, inflammasome formation and activation may be reduced via increased ASC methylation. ASC expression increases with age,[37] while moderate intensity aerobic exercise increases ASC methylation.[24] However these exercise-induced epigenetic changes have not been correlated with circulating cytokines, such as IL-1β or IL-18, nor has ASC methylation been assessed in persons with HF.

Attempts to Modulate Inflammasome

Strategies to alter inflammasome function in mice, such as genetic knockouts of inflammasome proteins and interleukin-1β, binding circulating proteins (e.g., IL-1 Trap), IL-1 receptor antagonist (e.g., anakinra), and siRNA silencing, have demonstrated reduced ventricular remodeling in myocardial infarction-induced or ischemic models of HF. Preliminary studies using anakinra post ST-segment elevation myocardial infarction in humans reduced the incidence of subsequent HF,[24,25] demonstrating that a reduction in IL-1β activity decreases ventricular remodeling. A two-week treatment with anakinra improved peak VO2 in persons with HFpEF in the absence of an exercise intervention.[44] A recent trial of an intermediate substrate in the synthesis of glyburide (16673-34-0) demonstrated inhibition of NLRP3 in a mouse model of acute myocardial infarction.[68] IL-18 binding protein (IL-18BP) has been shown to prevent systolic dysfunction in mice treated with plasma from decompensated HF patients.[69] Use of IL-18BP in rheumatoid arthritis (RA) treatment demonstrated only mild to moderate adverse events.[70] Anti-IL-18 antibodies are currently under investigation for treatment of type II diabetes mellitus, and may prove to be an effective therapy for HF. Because IL-1 family cytokines are important in innate immune processes, such as fever, treatment with IL-18 antibodies may be an important component of reducing deleterious inflammatory processes initiated by inflammasome activation with less immune suppression.

The main translational focus of immune modulation in HF has been on cytokine proteins, however, clinical trials targeting pro-inflammatory cytokines, such as anti-TNFα therapy, have yet to demonstrate improvements in HF outcomes and, in some cases, have proven to be harmful.[16,45] Efforts at immune modulation using disease-

modifying agents of rheumatoid diseases (DMARDs), such as anakinra and methotrexate, have demonstrated improvements for chronic inflammatory diseases, such as rheumatoid arthritis, gout, and recently post-infarction HF.[16] Potent anti-inflammatory therapy, anti-TNFα in particular, has a risk for infection.[16] Treatment of gram-positive sepsis with anti-TNFα therapy had a higher mortality rate than those not receiving treatment.[71] In contrast, treatment of IL-1 receptor antagonist for sepsis demonstrated treatment safety and dose-dependent efficacy with increased survival and decreased IL-6 and cytokine expression.[72] While trials in RA with anakinra demonstrated increased risk of upper respiratory infections, it should be noted that participants also took other immune suppressing drugs, including NSAIDs, corticosteroids, and other DMARDs. In fact, those who took anakinra without corticosteroids had a lower infection rate than participants not taking corticosteroids.

A large anti-cytokine study, The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS, www.thecantos.org), is currently examining if IL-1β inhibition via canakinumab, an IL-1β neutralizing monoclonal antibody, leads to decreased rates of recurrent MI, stroke, and cardiovascular death in persons with coronary artery disease and increased CRP. This study will enroll 17,500 participants who are post-AMI and have persistently elevated CRP.[73] Preliminary analysis of 556 participants examined the relationship between inflammation and atherosclerosis, and found that treatment with canakinumab decreased CRP over placebo, with no group differences in clinical adverse events.[74] This study may reveal a new approach to preventing ischemic HF through dampening the effects of inflammasome activation.

Anti-cytokine therapy may decrease cytokine levels below the physiologic levels needed for myocardial repair. Alternative methods, such as epigenetic regulation of inflammatory response and targeting upstream components of the signaling cascade have been proposed[16] however no trials in humans have been reported to date. The NLRP3 inflammasome has been shown to contribute to the development of HF after myocardial infarction.[5] ASC is a key component of the NLRP3 inflammasome and is necessary for caspase-1 mediated activation of IL-1β and IL-18. Inflammation in HF can be regulated by ASC methylation (Figure 2). This pathway is important to our understanding of the pathological processes behind myocardial remodeling in HF as our current understanding does not distinguish the individual contributions of the cytokines involved.

Future Directions

Historically anti-inflammatory therapies, e.g. NSAIDs and TNFα inhibitors infliximab and etanercept were not beneficial in HF.[8,44] While recent trials with other DMARDs, e.g. anakinra, have demonstrated prevention of remodeling post infarction, the over benefit of anti-inflammatory therapy in HF is unknown.[24,25] Anti-inflammatory therapy can lead to compromised host defense or amplification of inflammatory processes due to the many redundancies.[16] Further exploration of drug therapy that targets the inflammasome is needed. One new drug, 16673-34-0 was found to inhibit inflammasome formation post infarction in a mouse model.[68]

It is possible that epigenetic regulation of ASC activation in HF could dampen adverse inflammatory processes without disrupting cellular and tissue homeostasis. The involvement of the inflammasome components in various pathways makes finding

specific inflammasome targets a challenge as it may lead to unintended consequences. For example, by suppressing NALP3 function, it may be possible to also remove an important intracellular surveyor of danger signals. Some degree of inflammation is necessary for proper healing after insult or injury. The study by Nakajima et al.[31] demonstrated an increase in ASC methylation in older healthy adults after a moderate intensity aerobic exercise program. This study found that ASC methylation decreases with age and that these changes can be modified with moderate intensity aerobic exercise. While this study did not examine inflammatory markers related to levels of ASC methylation, regular aerobic exercise may prove to be an effective non-pharmacological modulator of inflammasome activation in HF.

A two-week treatment with anakinra, an IL-1 receptor antagonist, improved peak exercise oxygen consumption in HF in the absence of an exercise intervention.[44] Thus, aerobic capacity in HF may be related to increase circulating inflammatory cytokines and aerobic exercise reduces inflammatory cytokines. But the effect of aerobic exercise on ASC methylation and IL-18 in HF has not been previously examined. Aerobic exercise-induced ASC methylation may be a non-pharmacologic method of inflammasome modulation leading to decreased inflammation and improved outcomes in HF.

Conclusion

Attenuating HF disease progression by way of dampening inflammatory process is an ongoing area of intervention research with much promise. Considering the poor prognosis for HF patients, identification of novel inflammatory pathways and effectively intervening to slow HF progression is an important goal for HF research. Anti-

inflammatory drug therapy can lead to compromised host defense or further amplification of inflammatory processes due to the many redundancies and compensatory responses built into this complicated defense system. Targeting specific inflammatory pathways, such as NLRP3 activation, may provide a more precise approach to reducing deleterious inflammation in HF while leaving some innate host defense intact. Further research on targeting the NLRP3 inflammasome in HF patients is warranted.

References

1. Eslick GD, Thampan BV, Nalos M, McLean AS, Sluyter R. Circulating interleukin-18 concentrations and a loss-of-function P2x7 polymorphism in heart failure. 2008.

2. Van Tassell B, Varma A, Salloum FN, et al. Interleukin-1 trap attenuates cardiac remodeling after experimental acute myocardial infarction in mice. *J. Cardiovasc. Pharmacol.* 2010;22:117-122.

3. Heidenreich PA, Trogdon JG, Khavjou OA, et al. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation.* 2011;123(8):933-944.

4. Yancy CW, Jessup M, Bozkurt B, et al. 2013 ACCF/AHA guideline for the management of heart failure. A report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* 2013;epub ahead of print. doi:doi: 10.1016/j.jacc.2013.05.019

5. Abbate A. The heart on fire: Inflammasome and cardiomyopathy. *Exp. Physiol.* 2013;98(2):385.

6. Packer M. The neurohormonal hypothesis: a theory to explain the mechanism of disease progression in heart failure. *J. Am. Coll. Cardiol.* 1992;20(1):248-254.

7. Seta Y, Shan K, Bozkurt B, Oral H, Mann DL. Basic mechanisms in heart failure: the cytokine hypothesis. *J. Card. Fail.* 1996;2(3):243-249.

8. Chung ES, Packer M, Lo KH, Adedigbo A. Fasanmade, Willerson JT. Randomized, double-blind, placebo-controlled, pilot trial of Infliximab, a chimeric

monoclonal antibody to tumor necrosis factor-α, in patients with moderate-to-severe heart failure. Results of the Anti-TNF Therapy Against Cognestive Heart Failure (ATTACH) Trial. *Circulation.* 2003;107:3133-3140.

9. Hofmann U, Frantz S. How can we cure a heart ‘‘in flame’’? A translational view on inflammation in heart failure. *Basic Res. Cardiol.* 2013;108:356-375.

10. El-Menyar AA. Cytokines and myocardial dysfunction: state of the art. *J. Card. Fail.* 2008;14(1):61-74. doi:10.1016/j.cardfail.2007.09.006

11. von Haehling S, Schefold JC, Lainscak M, Doehner W, Anker SD. Inflammatory biomarkers in heart failure revisited: Much more than innocent bystanders. *Heart Failure Clinician.* 2009;5:549-560.

12. Bracey NA, Beck PL, Muruve DA, et al. The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1β. *Exp. Physiol.* 2013;98(2):462-472.

13. Mezzaroma E, Toldoa S, Farkasb D, et al. The inflammasome promotes adverse cardiac remodeling following acute myocardial infarction in the mouse. *PNAS.* 2011;108(49):19725-19730.

14. Kawaguchi M, Takahashi M, Hata T, et al. Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation.* 2011;123:594-604.

15. Nakayama H, Otsu K. Translation of hemodynamic stress to sterile inflammation in the heart. *Trends in endocrinology and metabolism: TEM.* Nov 2013;24(11):546-553. doi:10.1016/j.tem.2013.06.004

16. Tabas I, Glass CK. Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science.* 2013;336:166-172.

17. Otani H. The role of nitric oxide in myocardial repair and remodeling. *Antioxidant & Redox Signaling.* 2009;11(8):1913-1928.

18. Elahi M, Asopa S, Matata B. NO-cGMP and TNF-α counter regulatory system in blood: Understanding the mechanisms leading to myocardial dysfunction and failure. *Biochim. Biophys. Acta.* 2010;1772:5-14.

19. Vaduganathan M, Greene SJ, Butler J, et al. The immunological axis in heart failure: importance of the leukocyte differential. *Heart Fail Rev.* Nov 2013;18(6):835-845. doi:10.1007/s10741-012-9352-9

20. Campos JC, Gomes KMS, Ferreira JCB. Impact of exercise training on redox signaling in cardiovascular diseases. *Food Chem. Toxicol.* 2013;62:107-119.

21. Glezeva N, Baugh JA. Role of inflammation in the pathogenesis of heart failure with preserved ejection fraction and its potential as a therapeutic target. *Heart Failure Reviews.* 2013;epub ahead of print.

22. Schroder K, Zhou R, Tschopp J. The NLRP3 inflammasome: A sensor for metabolic danger? *Science.* 2010;327:296-300.

23. Kanneganti TD, M L, Nunez G. Intracellular NOD-like receptors in host defense and disease. *Immunity.* 2007;27:549-559.

24. Abbate A, Kontos MC, Grizzard JD, et al. Interleukin-1 blockade with Anakinra to prevent adverse cardiac remodeling after acute myocardial infarction (Virginia Commonwealth University Anakinra Remodeling Trial [VCU-ART] pilot study). *Am. J. Cardiol.* 2010;105:1371-1377.

25. Abbate A, Van Tassell BW, Biondi-Zoccai G, et al. Effects of interleukin-1 blockade with anakinra on adverse cardiac remodeling and heart failure after acute myocardial infarction [from the Virginia Commonwealth University-Anakinra Remodeling Trial (2) (VCU-ART2) pilot study]. *Am. J. Cardiol.* 2013;111(10):1394-1400.

26. Schroder K, Tschopp J. The inflammasome. *Cell Adhes. Commun.* 2010;140(821-832).

27. Lamkanfi M, Dixit VM. Inflammasomes and their roles in health and disease. *Annu. Rev. Cell Dev. Biol.* 2012;28:137-161. doi:10.1146/annurev-cellbio-101011-155745

28. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. *Nat. Biotechnol.* 2012;481:278-286. doi:10.1038/nature10759

29. Sutterwala FS, Haasken S, Cassel SL. Mechanism of NLRP3 inflammasome activation. *Ann. N. Y. Acad. Sci.* 2014. doi:10.1111/nyas.12458

30. Takahashi M. NLRP3 inflammasome as a novel player in myocardial infarction. *International Heart Journal.* 2014;55:101-105.

31. Nakajima K, Takeoka M, Mori M, et al. Exercise effects on methylation of ASC gene. *Int. J. Sports Med.* 2010;31:671-375.

32. Okamura H, Tsutsui H, Kashiwamura S-I, Yoshimoto T, Nakanishi K. Interleukin-18: A novel cytokinethat augments both innate and acquired immunity. *Adv. Immunol.* 1998;70:281-312.

33. Fedak PWM, Verma S, Weisel RD, Li R-K. Cardiac remodeling and failure. From molecules to Man (part 1). *Cardiovascular Pathology.* 2005;14:1-11. doi:10.1016/j.carpath.2001.12.002

34. Bauernfeind FG, Horvath G, Stutz A, et al. Cutting edge: NF-κB activating pattern recoginition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* 2009;183:787-791. doi:10.4049/jimmunol.901363

35. Tschopp J, Schroder K. NLRP3 inflammasome activation: the convergence of multiple signalling pathwayson ROS production? *Nature Reviews Immunology.* 2010;10:210-215.

36. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. *Cell.* 2014;157:1013-1022. doi:10.1016/j.cell.2014.04.007

37. Martinon F, Mayor A, Tschopp J. The inflammasomes: Guardians of the body. *Annu. Rev. Immunol.* 2009;27:229-265. doi:10.1146/annurev.immunol.021905.132715

38. Bracey NA, Gershkovich B, Chun J, et al. Mitochondrial NLRP3 induces reactive oxygen species to promote Smad signaling and fibrosis independent from the inflammasome. *J. Biol. Chem.* 2014. doi:0.1074/jbc.M114.550624

39. PvWilson S, Cassel SL. Inflammasome-mediated autoinflammatory disorders. *Postgrad. Med. J.* 2010;122(5):125-133. doi:10.3810/pgm.2010.09.2209

40. Taniguchi S, Sagara J. Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC. *Seminars in Immunopathology.* 2007;29:231-238.

41. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 2009;27:519-550.

42. Dinarello CA. A clinical perspective of IL-1b as the gatekeeper of inflammation. *Eur. J. Immunol.* 2011;41:1203-1217.

43. Van Tassell B, Seropian IM, Toldo S, Mezzaroma E, Abbate A. Interleukin-1b induces a reversible cardiomyopathy in the mouse. *Inflamm. Res.* 2013;62:637-640.

44. Van Tassell B, Arena RA, Toldo S, et al. Enhanced interleukin-1 activity contributes to exercise intolerance in patients with systolic heart failure. *PLoS ONE.* 2012;7(3):e33438.

45. Pomerantz BJ, Reznikov LL, Harken AH, Dinarello CA. Inhibition of caspase 1 reduces human myocardial ischemic dysfunction via inhibition of IL-18 and IL-1β. *Proc. Natl. Acad. Sci. U. S. A.* 2001;98(5):2871-2876.

46. Abbate A, Van Tassell B, Seropian IM, et al. Interleukin-1b modulation using a genetically engineered antibody prevents adverse cardiac remodelling following acute myocardial infarction in the mouse. *European Journal of Heart Failure.* 2010;12:319-322.

47. Dinarello CA, Simon A, van der Meer J. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nature Reviews Drug Discovery.* 2012;11:633-652.

48. Mallat Z, Heymes C, Corbaz A, et al. Evidence for altered interleukin (IL)-18 pathway in human heart failure. *The FASEB Journal.* 2004.

49. Yamaoka-Tojo M, Tojo T, Inomata T, Machida Y, Osada K, Izumi T. Circulating levels of interleukin 18 reflect etiologies of heart failure: Th1/TH2 cytokine imbalance

exaggerates the pathophysiology of advanced heart failure. *J. Card. Fail.* 2002;8(1):21-27.

50. Ueno N, Kashiwamura S-i, Ueda H, et al. Role of interleukin 18 in nitric oxide production and pancreatic damage during acute pancreatitis. *Shock.* 2005;24(6):564-570.

51. Umar S, van der Laarse A. Nitric oxide and nitric oxide synthase isoforms in the normal, hypertrophic and failing heart. *Mol. Cell. Biochem.* 2010;333:191-201.

52. Hedayat M, Mahmoudi MJ, Rose NR, Rezaei N. Proinflammatory cytokines in heart failure: double-edged swords. *Heart Failure Reviews.* 2010;15:543-562.

53. Bozkurt B, Torre-Amione G, Warren MS, et al. Results of targeted antitumor necrosis factor therapy with etanercept (ENBREL) in patients with advanced heart failure. *Circulation.* 2001;103:1044-1047.

54. Burkard T, Pfister O, Rickli H, et al. Prognostic impact of systemic inflammatory diseases in elderly patients with congestive heart failure. *QJM.* 2013;Epub ahead of print.

55. Chen W, Frangogiannis NG. Fibroblasts in post-infarction inflammation and cardiac repair. *Biochim. Biophys. Acta.* Apr 2013;1833(4):945-953. doi:10.1016/j.bbamcr.2012.08.023

56. Bracey NA, Gershkovich B, Chun J, et al. Mitochondrial NLRP3 protein induces reactive oxygen species to promote Smad protein signaling and fibrosis independent from the inflammasome. *J. Biol. Chem.* Jul 11 2014;289(28):19571-19584. doi:10.1074/jbc.M114.550624

57. Toldo S, Kannan H, Bussani R, et al. Formation of the inflammasome in acute myocarditis. *Int. J. Cardiol.* Feb 15 2014;171(3):e119-121. doi:10.1016/j.ijcard.2013.12.137

58. Haas J, Frese KS, Park YJ, et al. Alterations in cardiac DNA methylation in human dilated cardiomyopathy. *EMBO molecular medicine.* Mar 2013;5(3):413-429. doi:10.1002/emmm.201201553

59. Kaneda R, Takada S, Yamashita Y, et al. Genome-wide histone methylation profile for heart failure. *Genes Cells.* Jan 2009;14(1):69-77. doi:10.1111/j.1365-2443.2008.01252.x

60. Koczor CA, Lee EK, Torres RA, et al. Detection of differentially methylated gene promoters in failing and nonfailing human left ventricle myocardium using computation analysis. *Physiological genomics.* Jul 15 2013;45(14):597-605. doi:10.1152/physiolgenomics.00013.2013

61. Asrih M, Steffens S. Emerging role of epigenetics and miRNA in diabetic cardiomyopathy. *Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology.* Mar-Apr 2013;22(2):117-125. doi:10.1016/j.carpath.2012.07.004

62. Movassagh M, Choy MK, Goddard M, Bennett MR, Down TA, Foo RS. Differential DNA methylation correlates with differential expression of angiogenic factors in human heart failure. *PLoS One.* 2010;5(1):e8564. doi:10.1371/journal.pone.0008564

63. Montgomery RL, Davis CA, Potthoff MJ, et al. Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. *Genes Dev.* Jul 15 2007;21(14):1790-1802. doi:10.1101/gad.1563807

64. Angrisano T, Schiattarella GG, Keller S, et al. Epigenetic switch at atp2a2 and myh7 gene promoters in pressure overload-induced heart failure. *PLoS One.* 2014;9(9):e106024. doi:10.1371/journal.pone.0106024

65. Duygu B, Poels EM, da Costa Martins PA. Genetics and epigenetics of arrhythmia and heart failure. *Frontiers in genetics.* 2013;4:219. doi:10.3389/fgene.2013.00219

66. Pandya K, Smithies O. beta-MyHC and cardiac hypertrophy: size does matter. *Circ. Res.* Sep 2 2011;109(6):609-610. doi:10.1161/circresaha.111.252619

67. Majumdar G, Johnson IM, Kale S, Raghow R. Epigenetic regulation of cardiac muscle-specific genes in H9c2 cells by Interleukin-18 and histone deacetylase inhibitor m-carboxycinnamic acid bis-hydroxamide. *Mol. Cell. Biochem.* May 2008;312(1-2):47-60. doi:10.1007/s11010-008-9720-x

68. Marchetti C, Chojnacki J, Toldo S, et al. A novel pharmacologic inhibitor of the NLRP3 inflammasome limits myocardial injury after ischemia-reperfusion in the mouse. *J. Cardiovasc. Pharmacol.* 2014;63:316-322.

69. Toldo S, Mezzaroma E, O'Brien L, et al. Interleukin-18 mediates interleukin-1-induced cardiac dysfunction. *American journal of physiology. Heart and circulatory physiology.* Apr 1 2014;306(7):H1025-1031. doi:10.1152/ajpheart.00795.2013

70. O'Brien LC, Mezzaroma E, Van Tassell BW, et al. Interleukin-18 as a therapeutic target in acute myocardial infarction and heart failure. *Mol. Med.* 2014;20(1):221-229. doi:10.2119/molmed.2014.00034

71. Fisher CJ, Jr., Agosti JM, Opal SM, et al. Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. The Soluble TNF Receptor Sepsis Study

Group. *N. Engl. J. Med.* Jun 27 1996;334(26):1697-1702. doi:10.1056/nejm199606273342603

72. Fisher CJ, Jr., Slotman GJ, Opal SM, et al. Initial evaluation of human recombinant interleukin-1 receptor antagonist in the treatment of sepsis syndrome: a randomized, open-label, placebo-controlled multicenter trial. *Crit. Care Med.* Jan 1994;22(1):12-21.

73. Ridker PM, Thuren T, Zalewski A, Libby P. Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). *Am. Heart J.* Oct 2011;162(4):597-605. doi:10.1016/j.ahj.2011.06.012

74. Ridker PM, Howard CP, Walter V, et al. Effects of interleukin-1beta inhibition with canakinumab on hemoglobin A1c, lipids, C-reactive protein, interleukin-6, and fibrinogen: a phase IIb randomized, placebo-controlled trial. *Circulation.* Dec 4 2012;126(23):2739-2748. doi:10.1161/circulationaha.112.122556

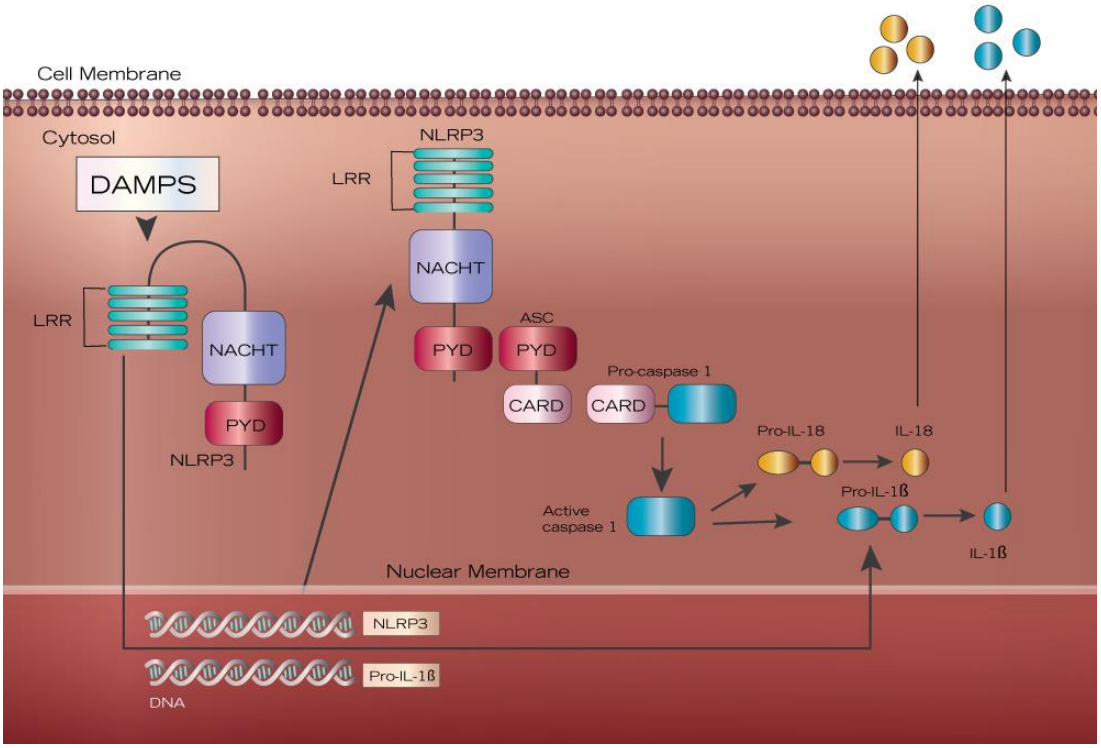
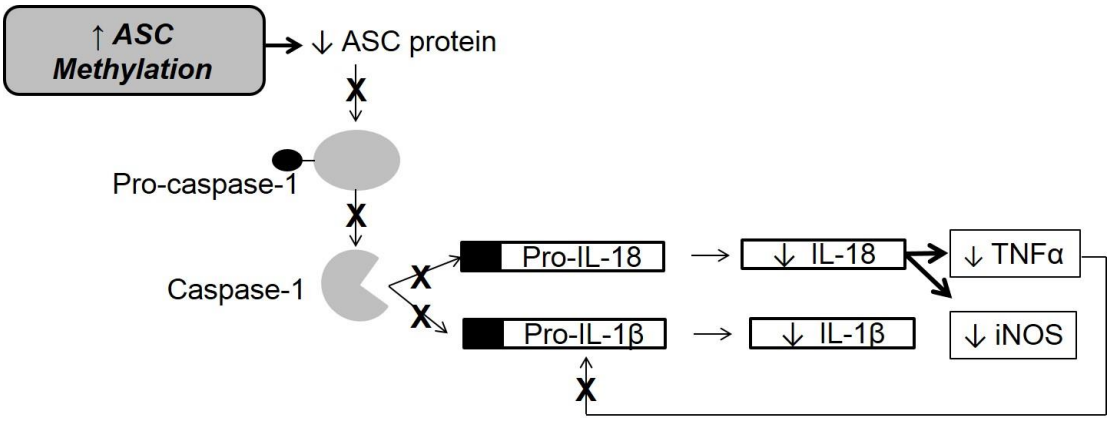


Figure 2.1: The NLRP3 Inflammasome NLRP3 inflammasome is comprised of three proteins: NLRP3, ASC and pro-caspase-1. NRLP3 has three domains: leucine-rich repeats (LRR), NACHT domain, and a PYD domain. The adaptor protein ASC pairs with NLRP3 via PYD domains and with pro-caspase-1 via CARD domains. DAMP (danger-associated molecular patterns) activation via LRR triggers transcription of NLRP3 and pro-IL-1β. The fully assembled NRLP3 inflammasome activates caspase-1, leading to the activation and release of IL-1β and IL-18.

Figure 2.2: Proposed Pathway of Epigenetic Regulation of the Inflammasome in Heart Failure



Chapter III

ASC methylation and interleukin-1β are associated with aerobic capacity in heart failure

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Keywords: aerobic capacity, inflammation, inflammasome, heart failure

Abstract

Background: Aerobic capacity, as measured by peak oxygen uptake ($\dot{V}O_2$), is one of the most powerful predictors of prognosis in heart failure (HF). Inflammation is a key factor contributing to alterations in aerobic capacity, and interleukin (IL)-1 family cytokines (IL-1β and IL-18) are implicated in this process. The adaptor protein ASC (apoptosis associated speck-like protein containing a caspase recruitment domain) is necessary for inflammasome activation of IL-1β and IL-18. ASC expression is controlled through epigenetic modification, and lower ASC methylation is associated with worse outcomes in HF.

Purpose: To examine the relationships between ASC methylation, IL-1β, and IL-18 with peak $\dot{V}O_2$ in persons with HF.

Methods: This cross-sectional study examined the relationship between ASC methylation and inflammatory cytokines (IL-1β, IL-18) with peak $\dot{V}O_2$ in 54 stable outpatients with HF on optimal medical therapy. All participants were NYHA class II or III, not currently engaged in an exercise program, and physically able to complete a symptom limited modified Balke exercise treadmill test.

Results: Participants were 59± 10 years of age, 52% female, and 59% African American. Mean peak $\dot{V}O_2$ was 16.68 ± 4.7 ml/kg/min. Peak $\dot{V}O_2$ was positively associated with mean percent ASC methylation (r=.47, p=.001) and negatively associated with IL-1β (r=-.38, p=.007). A negative association was found between peak $\dot{V}O_2$ and IL-18 (r=.4, p=.044) only among those with reduced ejection fraction (<40%). Multiple linear regression models controlling for left ventricular ejection fraction and gender demonstrated that peak $\dot{V}O2$ increased by 2.73 ml/kg/min for every 1% increase in mean

ASC methylation and decreased by 2.15 ml/kg/min for every 1 pg/mL increase in plasma IL-1β.

Conclusions: Mean percent ASC methylation and plasma IL-1β levels are associated with clinically meaningful differences in peak $\dot{V}O_2$ in persons with HF. Inflammasome activation may play a mechanistic role in determining aerobic capacity. ASC methylation is a potentially modifiable mechanism for reducing the inflammatory response, thereby improving aerobic capacity in HF. Future research examining modification of ASC expression, such as exercise interventions that increase ASC methylation, may improve aerobic capacity in persons with HF.

Introduction

Reduced exercise capacity is a characteristic symptom of heart failure (HF), and is accompanied by dyspnea, fatigue, and muscle weakness, even during low-intensity exercise.[1,2] This exercise intolerance is associated with reduced aerobic capacity and low ventilatory efficiency, leading to decreased functional capacity and poor quality of life.[2,3] Clinical evaluation of aerobic capacity in HF is measured by peak oxygen uptake ($\dot{V}O_2$), a measure of oxygen consumption during a maximum effort treadmill test, and is a strong prognostic indicator of decompensation and mortality.[4,5] Aerobic capacity is a well-established and powerful predictor of prognosis in persons with HF.[6]

Aerobic capacity is determined by the integrity of the cardiovascular, respiratory, and skeletal muscle systems, all of which are diminished in HF.[1,7-9] Inflammation negatively affects cardiac, respiratory, and skeletal muscle pathophysiology.[9-12] Chronic inflammation and increased circulating cytokines contribute to the pathophysiology and worsening of HF by altering cardiac structure and function.[13] These inflammatory cytokines also play a role in altering skeletal muscle function due to their catabolic effects.[10] In addition, chronic inflammation in HF leads to altered breathing patterns, such as inspiratory muscle weakness.[14] Thus, inflammation is a key component of pathophysiological changes that lead to decreased aerobic capacity in persons with HF.

Inflammation and Peak $\dot{V}O_2$

Aerobic capacity is negatively associated with inflammatory cytokines, in both healthy populations and in disease states, such as HF.[15] Further, changes in peak $\dot{V}O_2$ in response to an intervention are accompanied by changes in systemic inflammatory

cytokines, such as c-reactive protein (CRP).[15,16] A two-week treatment with the IL-1 cytokine blocker anakinra demonstrated a reduction in systemic inflammation and improvement in peak $\dot{V}O_2$ in persons with HF in the absence of an exercise intervention.[17,18] Thus, aerobic capacity in HF may be related to increased circulating IL-1 family inflammatory cytokines.

The IL-1 family cytokines, IL-1β and IL-18, are activated by the inflammasome, a complex of intracellular interaction proteins that triggers maturation of cytokines to initiate and amplify the inflammatory response.[23-25] The inflammasome is composed of a NOD (nucleotide-binding oligomerization domain)-like receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and pro-caspase-1.[26-28] The activated inflammasome cleaves pro-caspase-1 into the active enzyme caspase-1.[29] Caspase-1, in turn, activates IL-1 family cytokines IL-1β and IL-18, by cleavage of pro-IL-1β and pro-IL-18 into active forms.[24,30-32]

The adaptor protein ASC recruits pro-caspase-1 to the inflammasome complex and is necessary for caspase-1 mediated activation of IL-1β and IL-18.[19-22] ASC expression is controlled by epigenetic modification via methylation of a CpG island in the promoter region of exon 1.[23-26] Increased methylation of ASC is associated with decreased plasma IL-1β in persons with HF.[27]

Higher percent ASC methylation and lower ASC mRNA expression are positively associated with the six-minute walk test (6MWT) in persons with HF.[27] Although the 6MWT has been shown to be a reliable submaximal measure of functional capacity,[28] a measure of maximal aerobic capacity can provide better insight into the functional capacity of an individual with HF. The purpose of this study, therefore, was to examine

the relationships between ASC methylation, IL-1β, and IL-18 with peak $\dot{V}O_2$ in persons with HF.

Methods

Study Design

This cross-sectional study examined the relationship between ASC methylation and peak $\dot{V}O_2$ in stable outpatients with HF. Fifty-four participants were enrolled from 1 of 4 large urban tertiary-care hospitals that had multidisciplinary outpatient HF clinics.

Study Sample

Participants were screened for eligibility using electronic medical record review. Inclusion criteria included: documented medical diagnosis of NYHA class II or III, aged 40-75 years; left ventricular ejection fraction (LVEF) ≥10% documented within the last year by echocardiogram, cardiac catheterization ventriculography, or radionuclide ventriculography; and receiving medication therapy for HF according to the American College of Cardiology/American Heart Association recommendation guidelines, including angiotensin-converting-enzyme inhibitors or angiotensin receptor blockers, beta blockers, and diuretics for participants with reduced ejection fraction (LVEF ≤ 40%)[29] for at least 8 weeks prior to study enrollment. Exclusion criteria included: medical diagnosis of NYHA class I or IV; change in HF therapy within the previous 8 weeks; worsening HF symptoms within the last 5 days; unstable angina; renal insufficiency (serum creatinine > 3.0 mg/dL); fixed rate pacemaker; uncontrolled hypertension, involved in any structured exercise program or exercising 3 or more times

per week for a minimum of 30 minutes; hospitalization within the previous 30 days; and any disorder precluding an exercise treadmill test.

Severity of illness was controlled by limiting participants to NYHA class II and III, who are more similar in response to exercise than class I and IV. In addition, severity of illness was controlled by LVEF limitations (LVEF > 10%) and optimal medication therapy for HF. The age range was selected to avoid confounding effects of age and sarcopenia with the outcome, aerobic capacity; participants below the age of 40 years are likely to have HF for other reasons than the majority of the general HF population.[30] Older age (>75) is associated with reduced exercise capacity which may confound the outcome measurement of peak $\dot{V}O_2$.[31] In addition, persons over the age of 75 are at higher risk for adverse events during exercise treadmill testing.[32]

Study Protocol

All studies were performed under research protocols approved by the Institutional Review Boards of Emory University and participating institutions. Each subject was informed of testing protocols and the potential risks and benefits of participation. All participants provided written consent before participation. Blood samples were collected in the morning after an overnight fast. Blood was collected in a vacutainer with EDTA, separated into plasma and buffy coat, and stored at -80°C until analysis.

Measurements

Demographic and Clinical Data

Sociodemographic and clinical variables included age, gender, race, medical history, and medications, and were obtained from medical records and a self-report questionnaire. The Charlson Comorbidity Index (CCI)[33] was used to assess for other chronic conditions. Height was measured with a standard stadiometer, without shoes and recorded in centimeters. Weight was measured in kilograms using a calibrated scale with the participant in light clothing, without shoes. Body mass index (BMI) was calculated by the formula: BMI = (weight in kg)/(height in cm)^2. Participants with LVEF <40% were categorized as heart failure with reduced ejection fraction (HFrEF), and those with LVEF ≥40% were categorized as preserved ejection fraction (HFpEF).[29]

Six Minute Walk Test

The six-minute walk test (6MWT) was performed along a 30-meter marked level hallway. Participants were asked to walk between two marked points using a standardized protocol.[28] The distance walked in meters over a measured 6-minutes was recorded.

Cardiopulmonary Exercise Stress Test

Aerobic capacity was assessed using the modified Balke maximal symptom-limited treadmill test[34,35] to determine peak oxygen consumption (peak $\dot{V}O_2$). The treadmill test was performed on the same visit as the 6MWT, however participants were allowed to rest for an hour after the 6MWT before beginning the treadmill protocol. Continuous gas exchange (VMAX Spectra 29 CPET Instrument, Yorba Linda, CA), telemetry, blood pressure, rating of perceived exertion, and oxygen saturation were

assessed for each patient 1 minute before, continuously during exercise, and 4 minutes after the exercise test according to American Heart Association guidelines.[3] The test protocol was as follows: 0%incline at 2.0 mph on motorized treadmill for 3 minutes with an increase in incline of 3.5% every 3 minutes for 18 minutes. At 18 minutes, speed increased to 3.0 mph and incline decreased to 12.5%. No participants progressed beyond this point. Participants who reached a respiratory quotient (RQ; $\dot{V}CO_2/\dot{V}O_2$) $\geq$1 at peak $\dot{V}O_2$ were considered to have attained anaerobic threshold.[36]

ASC Methylation

Percent methylation of 7 CpG sites in the intron region of ASC was measured as previously reported.[27] In brief, genomic DNA from peripheral blood mononuclear cells (PBMCs) was bisulfite treated and amplified by PCR followed by pyrosequencing for methylation quantification.[37] Methylation of 7 CpG sites in the promoter region of exon 1 were measured[27] and analyzed as mean percent methylation. The mean percent methylation of the 7 CpG sites for each individual was calculated as the sum of percent methylation of the CpG sites divided by 7.

Cytokines

IL-1β and IL-18 were analyzed from plasma that has been separated from collected whole blood and stored at -80°C immediately after collection. Plasma cytokines were measured in duplicate using commercially available ELISA kits (eBioscience). Plates were read on a BioTek microplate reader and analyzed using Gen5

software. Curve fitting was selected among linear, quadratic and 4-point based on the best regression coefficient.

Data Analysis

All data were reviewed for data entry errors, potential outliers, and missing data. Clinical and demographic data were complete, and only participants who completed the study protocol (blood draw, 6MWT, treadmill test, and physical examination) were included in the analysis. Distributions were assessed for deviations from normality. IL-18 did not meet criteria for normality and was log transformed (LN) for statistical analysis. However, non-transformed IL-18 values are presented in descriptive data. Descriptive statistics were used to calculate means and standard deviations or percentages for demographic and clinical data. Pearson correlation analysis was performed to identify linear associations between variables and identify covariates for further analysis. An unpaired Student's t test was used to determine differences in measures between demographic groups. Linear regression analysis was performed to examine linear relationships between the dependent variable, aerobic capacity (as measured by peak $\dot{V}O_2$), and the independent variables, ASC methylation and cytokines, controlling for covariates. Linear regression analysis was performed using 2 models. Model 1 consisted of the independent and dependent variables, without adjusting for covariates. Model 2 also included covariates (age, gender, LVEF, 6MWT, BMI and medications) based on previous documentation and performance of the data and relationships within this study. The variables included in the final model were selected using stepwise selection with an

alpha set at 0.10 to minimize multicollinearity and choose the most parsimonious final model. All analysis was performed using SPSS version 23.

Results

Patient Characteristics

Demographic and clinical data are presented in Table 1. Most participants (52%) were female, and 59.3% were African American. LVEF ranged from 15 to 65%, and 63.0% of the participants had LVEF <40%. The most common comorbidities were hypertension (N=36, 66.7%), dyslipidemia (N=30, 55.6%), depression (N=20, 37.0%), and diabetes (N=19, 35.2%). One-third of participants (N=16, 29.6%) had a previous MI. Age was positively associated with LVEF (r=.281, p=.044), and females had higher LVEF as compared to males (38.23 ± 14.9 vs. 29.44 ± 13.6, respectively; t=2.26, p=.03).

Physical Measures

Six-minute walk test total distanced ranged from 168 – 492 meters, with 24.1% (N=13) of participants walking < 300 meters, a level associated with mortality risk in HF[38] (Table 2). Total treadmill time ranged from 1.8 – 18.3 minutes and peak $\dot{V}O_2$ ranged from 7.6 – 29.9 ml/kg/min. Males had higher treadmill duration and peak $\dot{V}O2$ as compared to females (Table 2). In addition, males had higher RQ than females (Table 2), and a higher proportion of males met anaerobic threshold than females (42% vs 10%, respectively). There was a negative association between 6MWT and age (r=-.46, p=<.001). Peak $\dot{V}O_2$ was positively associated with total treadmill time (r=.58, p=<.001),

6MWT total distance (r=.29, p=.05), and negatively associated with LVEF (r=-.32, p=.02) and BMI (r=-.31, p=.03).

ASC and Cytokines

ASC and cytokine data are presented in Table 3. Mean percent ASC methylation ranged from 1.33 – 6.82 and was not associated with age (r=-.06, p=.69), gender (r=.04, p=.81), or race (r=.19, p=.21). ASC methylation was negatively associated with IL-1β (r=-.44, p=.001), but not IL-18 (r=-.07, p=.64). IL-18 was lower among black participants as compared to non-black participants (141.42 ± 46.9 vs. 186.47 ± 59.8, respectively; t=-3.15, p=.003).

Peak $\dot{V}O_2$ was positively associated with mean percent ASC methylation (r=.47, p=.001) and negatively associated with IL-1β (r=-.38, p=.007). No association with aerobic capacity and IL-18 was found. The sample was dichotomized into HFrEF (n=34, 63%) and HFpEF (n=20, 37%) to analyze differences in relationships among the outcome variables in these clinically different groups. For those categorized as HFrEF, peak $\dot{V}O_2$ was positively associated with mean percent ASC methylation (r=.65, p<.001), and negatively associated with IL-1β (r=-.49, p=.006) and IL-18 (r=-.40, p=.044), controlling for gender. Among those categorized as HFpEF, peak $\dot{V}O_2$ was associated with mean percent ASC methylation (r=.76, p=.006) and IL-1β (r=-.45, p=.048), but not IL-18, when controlling for gender. The relationship between ASC methylation and IL-1β remained significant for both HFrEF (r=-.60, p<.001) and HFpEF (r=-.74, p<.001).

A multiple linear regression was performed to predict peak $\dot{V}O_2$ based on mean percent ASC methylation and plasma IL-1β levels, controlling for covariates (Table 4).

The models demonstrated that peak V̇O2 increased by 2.30 ml/kg/min for every 1% increase in mean ASC methylation and decreased by 1.91 ml/kg/min for every 1 pg/mL increase in plasma IL-1β, adjusting for LVEF and gender. Gender remained a significant predictor of aerobic capacity in both models, such that male gender contributed to 2.42 ml/kg/min and 2.62 ml/kg/min in the ASC and IL-1β models, respectively. A sub-analysis was performed using only those participants who reached anaerobic threshold (RQ ≥ 1) to determine if these relationships remained significant among those who performed maximal effort. Participants who reached anaerobic threshold (n=14, 26%) during the treadmill test were mostly male (n=11, 79%). Linear regression analysis, controlling for LVEF, was repeated with the 14 participants in the anaerobic threshold sub-group. Because this was a mostly male population, gender was not included as a covariate. Significant linear relationships remained between peak $\dot{V}O_2$ and ASC methylation (β=11.14; 95% CI 3.93, 18.36; p=.006) and between peak $\dot{V}O_2$ and IL-1β (β=-2.02; 95% CI -4.46, -0.04; p=.021).

Discussion

This study demonstrated that the inflammasome pathway may impact aerobic capacity in HF patients and serves to broaden our understanding of the biological determinants of aerobic exercise capacity. Previously, we reported a positive linear association between ASC methylation and 6MWT total distance in a different population of persons with HF.[27] Here, we show that ASC methylation is positively related to peak $\dot{V}O_2$. In addition, IL-1β had a negative relationship with peak $\dot{V}O_2$, suggesting the inflammasome may play a mechanistic role in aerobic capacity in HF. Studies of short-

term administration of IL-1β blockade (anakinra) were associated with an increase in peak $\dot{V}O_2$ in persons with both HFrEF[39] and HFpEF.[17] Analysis of a small subset in the HFrEF study (n=3) demonstrated a decrease in IL-1β with anakinra treatment.[39] However, this analysis was too small to determine statistical significance and was not compared to changes in peak $\dot{V}O_2$. Nonetheless, the changes in peak $\dot{V}O_2$ after IL-1β blockade implicate this interleukin-1 family cytokine in the pathophysiology of decreased aerobic capacity in HF.

Mean percent ASC methylation was 5.84 ± 0.78, which is similar to our previous results in a different HF population.[27] The positive association of ASC methylation and peak $\dot{V}O_2$ in this study adds a putative mechanism of epigenetic control of inflammatory gene expression contributing to decreased aerobic capacity in HF. Increased ASC methylation is associated with decreased ASC protein and mRNA expression in HF.[27] Further, decreased ASC protein expression is associated with decreased IL-1β in HF.[27] In this study, linear regression analysis revealed that mean percent ASC methylation and plasma IL-1β levels are associated with clinically meaningful changes in peak $\dot{V}O_2$ in persons with HF. Together, these data suggest inflammasome activation of IL-1β plays a mechanistic role in physiological processes in HF.

The relationship between peak $\dot{V}O_2$ and IL-18 only in the HFrEF subset was surprising, and may reflect the role of IL-18 in the pathophysiology of HFrEF. Circulating IL-18 is significantly greater among those categorized as NYHA class IV as compared to class II and III, in HFrEF as compared to HFpEF, and in decompensated HF as compared to stable HF.[27,40,41] However, studies examining IL-18 in HF have primarily focused on post-ischemic ventricular changes or dilated cardiomyopathy related to

systolic dysfunction. IL-18 is regulated by an endogenous inhibitor, IL-18 binding protein (IL-18BP), which inactivates IL-18 when bound.[42] Gene expression of IL-18, its receptor IL-1Rα, and IL-18BP were found to be similar among persons with both mild and severe dilated cardiomyopathy.[41] This suggests that alterations in the IL-18/IL-18BP pathway occur early in the pathophysiological processes of HF, but are reflective of an HFrEF phenotype. HFpEF is caused by multiple impairments, related to factors such as diastolic reserve function, heart rate reserve, rhythm abnormalities, atrial dysfunction, and stiffening of the ventricles and vasculature.[43-46] The cellular changes in the myocardium alter structure and function differently in HFpEF and HFrEF, leading to different phenotypes.[47] Thus, while inflammatory processes are upregulated in both HFpEF and HFrEF, it is likely that specific inflammatory pathways differ. Overall, studies comparing biomarkers of HFpEF and HFrEF are limited. Further examination of changes in gene expression and signaling pathways may provide insights into the pathophysiology and new targets for treatment in this poorly understood syndrome.

Increased ASC methylation was associated with decreased plasma IL-1β. No association was found with ASC methylation and plasma IL-18. These findings are similar to our findings in a previous study,[27] and are likely indicative of redundancies in function and activation of IL-18.[48] Unlike IL-1β, IL-18 is not involved in the acute phase response and does not induce systemic inflammatory events, such as neutropenia and fever.[49,50] Monocytes can differentially secrete IL-18 or IL-1β, in response to specific signaling, such as leptin.[51] Further, while IL-1β activation is tightly regulated by the inflammasome,[52] IL-18 can be activated by non-canonical pathways (caspase-3 and Fas-induced caspase-8) and is regulated by the endogenous inhibitor, IL-18BP.[48,53-56] Thus,

IL-18 may represent a common signal downstream of different inflammatory pathways, and may occasionally function independent of IL-1β activity. Nonetheless, mechanistic and tissue-level studies may provide more insight into this relationship.

IL-18 was higher among white participants in this HF population. This has been reported previously in a population of persons with type 2 diabetes.[57] Although no mechanism for racial differences in IL-18 expression has been found, genetic and anthropometric differences have been suggested.[57] In HF, comorbidities and past ischemic events may drive differences in levels of circulating IL-18. In this study of persons with HF, a higher proportion of white participants had a previous history of myocardial infarction (MI) and dyslipidemia as compared to AA/black participants. Cardiac cells produce IL-18 in response to ischemia-reperfusion injury, and IL-18 is higher in persons with ischemic cardiomyopathy as compared to those with dilated cardiomyopathy.[40] IL-18 is involved in the process of atherogenesis and is associated with vascular events, although the mechanism has yet to be uncovered.[58] Further work is needed to determine the implications of these differences.

Gender differences in HF presentation have been previously reported.[59] In particular, females with HF, tend to have higher LVEF and lower peak $\dot{V}O_2$ than males.[59] In this study, the effects of gender and LVEF on peak $\dot{V}O_2$ were accounted for in the regression analysis. The relationships between ASC methylation and IL-1β with peak $\dot{V}O_2$ remained significant when controlling for both gender and LVEF. Thus, ASC methylation and IL-1β appear to influence peak $\dot{V}O_2$ independent of gender and LVEF.

A previous study by Nakajima *et al*.[37] found no relationship between ASC methylation and $\dot{V}O_{2max}$ in their study of healthy adults. This difference in findings

reported in the current study may be related to the pathophysiological and structural changes that limit aerobic capacity in HF. In HF, aerobic capacity is measured as peak $\dot{V}O_2$, as opposed to the $\dot{V}O_{2max}$ measurement in the healthy population. The measurement of $\dot{V}O_{2max}$ requires an individual to reach a plateau of maximum volume of oxygen used; persons with HF are rarely able to reach and sustain this plateau. Peak $\dot{V}O_2$ is reduced primarily by impaired cardiac output in both heart failure with reduced ejection fraction (HFrEF) and preserved ejection fraction (HFpEF).[2] Other factors such as endothelial dysfunction leading to vasoconstriction and decreased capacity for aerobic metabolism contribute to the reduction in maximum oxygen uptake in HF.[2] These physiological processes are amplified by a positive feedback loop of inflammation and may explain, at least in part, the association between peak $\dot{V}O_2$ and the inflammasome-related measures. Further investigation comparing these relationships to age and gender matched healthy controls is needed.

Increased cytokines in HF may contribute to the pathophysiology and disease progression by altering cardiac structure and function. Inflammatory cytokines may also contribute to peripheral alterations in vascular and skeletal muscle function. The cardiovascular, respiratory, and skeletal muscle systems all influence aerobic capacity,[2] and the individual contributions of pathophysiological changes in each system to impaired aerobic capacity are difficult to discern. Here we show the relationship between IL-1β and peak $\dot{V}O_2$ in a systemic source (plasma), but further investigation into the relationships and mechanisms of inflammatory cytokines in cardiovascular and skeletal muscle tissue is warranted.

Low aerobic capacity is a powerful predictor of premature morbidity and higher mortality in HF,[6] and adding IL-1β levels may provide better prediction of adverse outcomes in HF. Previously we demonstrated that ASC methylation, protein, and mRNA expression are predictors of clinical events in HF.[27] Taken together, this further implicates the inflammasome pathway in the pathophysiological processes of HF. Modification of inflammation via epigenetic modulation may be a novel target for intervention in HF.

Moderate intensity aerobic exercise, such as a walking program, has been shown to increase ASC methylation,[37] however these exercise-induced epigenetic changes have not been correlated with circulating cytokines, such as IL-1β or IL-18, or assessed in persons with HF. Aerobic exercise has been shown to be beneficial for most HF patients by altering the deleterious peripheral and central mechanisms, such as inflammatory cytokines, that contribute to HF exacerbations, worsened symptom severity, and poor clinical outcomes.[60-64] In addition, aerobic exercise reduces vascular resistance and improves endothelial function as well as the oxidative capacity of peripheral muscles, without worsening left ventricular remodeling.[62,64,65] The HF-ACTION trial[66] established the safety and efficacy of moderate-intensity aerobic exercise in patients with stable HF. Aerobic exercise has been shown to reduce inflammatory cytokines, but further studies are needed to establish the effect of aerobic exercise on ASC methylation and IL-1β in persons with HF.

Limitations

This study was cross-sectional with a relatively small sample size of 54 participants. A larger study following changes in $\dot{V}O_2$ and ASC methylation over time may shed more light on the dynamic relationship between these measures. Based on analysis of individual respiratory quotient values at reported peak $\dot{V}O_2$, a large proportion of participants stopped the treadmill test before depleting their cardiac reserve. Thus, the $\dot{V}O_2$ values reported here may not reflect true maximal oxygen consumption. However, due to the effects of inflammation on skeletal muscle dysfunction, and thus decreased aerobic capacity, it is possible that these participants indeed reached peak cardiac reserve exhaustion and IL-1β and ASC methylation are markers of this decreased capacity.

ASC methylation and cytokine analysis was performed using collected whole blood. While peripheral blood mononuclear cells (PBMCs) and serum cytokine levels are indicative of systemic inflammation, including cardiac and/or skeletal muscle biopsy samples would provide more insight into localized inflammatory changes affecting aerobic capacity. Further, healthy controls were not included in the study for comparison.

Conclusion

Mean percent ASC methylation and plasma IL-1β levels are associated with peak $\dot{V}O_2$ in persons with HF. This suggests inflammasome activation may play a role in determining aerobic capacity in persons with HF. ASC methylation is a potentially modifiable mechanism of reducing inflammation and improving functional capacity in HF. Future research examining modification of ASC expression, such as exercise

interventions that increase ASC methylation, may improve aerobic capacity in persons with HF.

References

1. Wong E, Selig S, Hare DL. Respiratory muscle dysfunction and training in chronic heart failure. *Heart Lung Circ.* May 2011;20(5):289-294. doi:10.1016/j.hlc.2011.01.009
2. Arena R, Cahalin LP, Borghi-Silva A, Phillips SA. Improving functional capacity in heart failure: the need for a multifaceted approach. *Curr. Opin. Cardiol.* Sep 2014;29(5):467-474. doi:10.1097/hco.0000000000000092
3. Balady GJ, Arena R, Sietsema K, Myers J, Coke L, Fletcher GF, et al. Clinician's Guide to cardiopulmonary exercise testing in adults: a scientific statement from the American Heart Association. *Circulation.* 2010;122:191-225.
4. Szlachcic J, Massie BM, Kramer BL, Topic N, Tubau J. Correlates and prognostic implication of exercise capacity in chronic congestive heart failure. *Am. J. Cardiol.* Apr 1 1985;55(8):1037-1042.
5. Cohn JN, Johnson GR, Shabetai R, Loeb H, Tristani F, Rector T, et al. Ejection fraction, peak exercise oxygen consumption, cardiothoracic ratio, ventricular arrhythmias, and plasma norepinephrine as determinants of prognosis in heart failure. The V-HeFT VA Cooperative Studies Group. *Circulation.* Jun 1993;87(6 Suppl):Vi5-16.
6. Bowen TS, Eisenkolb S, Werner S, Schwarzer M, Schuler G, Adams V. Inheriting a high aerobic fitness predisposes to skeletal muscle and endothelial dysfunction in chronic heart failure. *Int. J. Cardiol.* Jan 15 2016;203:353-356. doi:10.1016/j.ijcard.2015.10.125

7. Myers J, Gujja P, Neelagaru S, Burkhoff D. Cardiac output and cardiopulmonary responses to exercise in heart failure: application of a new bio-reactance device. *J. Card. Fail.* Oct 2007;13(8):629-636. doi:10.1016/j.cardfail.2007.05.009

8. Okita K, Yonezawa K, Nishijima H, Hanada A, Ohtsubo M, Kohya T, et al. Skeletal muscle metabolism limits exercise capacity in patients with chronic heart failure. *Circulation.* Nov 3 1998;98(18):1886-1891.

9. Gielen S, Adams V, Mobius-Winkler S, Linke A, Erbs S, Yu J, et al. Anti-inflammatory effects of exercise training in the skeletal muscle of patients with chronic heart failure. *J. Am. Coll. Cardiol.* Sep 3 2003;42(5):861-868.

10. Anker SD, Ponikowski PP, Clark AL, Leyva F, Rauchhaus M, Kemp M, et al. Cytokines and neurohormones relating to body composition alterations in the wasting syndrome of chronic heart failure. *Eur. Heart J.* May 1999;20(9):683-693. doi:10.1053/euhj.1998.1446

11. Van Tassell BW, Raleigh JM, Abbate A. Targeting interleukin-1 in heart failure and inflammatory heart disease. *Current heart failure reports.* Feb 2015;12(1):33-41. doi:10.1007/s11897-014-0231-7

12. Gosselin LE, Barkley JE, Spencer MJ, McCormick KM, Farkas GA. Ventilatory dysfunction in mdx mice: impact of tumor necrosis factor-alpha deletion. *Muscle Nerve.* Sep 2003;28(3):336-343. doi:10.1002/mus.10431

13. Mann DL. Innate immunity and the failing heart: the cytokine hypothesis revisited. *Circ. Res.* Mar 27 2015;116(7):1254-1268. doi:10.1161/circresaha.116.302317

14. Kusunose K, Mehra R. Targeting Sleep Disordered Breathing to Prevent Heart Failure: What is the Evidence? *Current cardiovascular risk reports.* Oct 1 2014;8(10):403. doi:10.1007/s12170-014-0403-8

15. Toth MJ, Ades PA, Tischler MD, Tracy RP, LeWinter MM. Immune activation is associated with reduced skeletal muscle mass and physical function in chronic heart failure. *Int. J. Cardiol.* May 10 2006;109(2):179-187. doi:10.1016/j.ijcard.2005.06.006

16. Santos RV, Viana VA, Boscolo RA, Marques VG, Santana MG, Lira FS, et al. Moderate exercise training modulates cytokine profile and sleep in elderly people. *Cytokine.* Dec 2012;60(3):731-735. doi:10.1016/j.cyto.2012.07.028

17. Van Tassell BW, Arena R, Biondi-Zoccai G, McNair Canada J, Oddi C, Abouzaki NA, et al. Effects of interleukin-1 blockade with anakinra on aerobic exercise capacity in patients with heart failure and preserved ejection fraction (from the D-HART pilot study). *Am. J. Cardiol.* Jan 15 2014;113(2):321-327. doi:10.1016/j.amjcard.2013.08.047

18. van Tassell B, Arena RA, Toldo S, Mezzaroma E, Azam T, Seropian IM, et al. Enhanced interleukin-1 activity contributes to exercise intolerance in patients with systolic heart failure. *PLoS ONE.* 2012;7(3):e33438. doi:10.1371/journal.pone.0033438

19. Abbate A. The heart on fire: Inflammasome and cardiomyopathy. *Exp. Physiol.* 2013;98(2):385. doi:10.1113/expphysiol.2012.069021

20. Bracey NA, Beck PL, Muruve DA, Hirota SA, Guo J, Jabagi H, et al. The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy

through interleukin-1β. *Exp. Physiol.* 2013;98(2):462-472. doi:10.1113/expphysiol.2012.068338

21. Lebel-Binay S, Berger A, Zinzindohoué F, Cugnenc P-H, Thiounn N, Fridman WH, et al. Interleukin-18: Biological properties and clinical implications. *Eur. Cytokine Netw.* 2000;11(1):15-26.

22. Taniguchi S, Sagara J. Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC. *Seminars in Immunopathology.* 2007;29:231-238.

23. Conway KE, McConnell BB, Bowring CE, Donald CD, Warren ST, Vertino PM. TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. *Cancer Res.* Nov 15 2000;60(22):6236-6242.

24. Stimson KM, Vertino PM. Methylation-mediated silencing of TMS1/ASC is accompanied by histone hypoacetylation and CpG island-localized changes in chromatin architecture. *J. Biol. Chem.* Feb 15 2002;277(7):4951-4958. doi:10.1074/jbc.M109809200

25. Levine JJ, Stimson-Crider KM, Vertino PM. Effects of methylation on expression of TMS1/ASC in human breast cancer cells. *Oncogene.* May 29 2003;22(22):3475-3488. doi:10.1038/sj.onc.1206430

26. Stone AR, Bobo W, Brat DJ, Devi NS, Van Meir EG, Vertino PM. Aberrant methylation and down-regulation of TMS1/ASC in human glioblastoma. *Am. J. Pathol.* Oct 2004;165(4):1151-1161. doi:10.1016/s0002-9440(10)63376-7

27. Butts B, Gary RA, Dunbar SB, Butler J. Methylation of Apoptosis-Associated Speck-Like Protein With a Caspase Recruitment Domain and Outcomes in Heart Failure. *J. Card. Fail.* Dec 14 2015. doi:10.1016/j.cardfail.2015.12.004

28. Bittner V, Weiner DH, Yusuf S, Rogers WJ, McIntyre KM, Bangdiwala SI, et al. Prediction of mortality and morbidity with a 6-minute walk test in patients with left ventricular dysfunction. SOLVD Investigators. *JAMA.* Oct 13 1993;270(14):1702-1707.

29. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey Jr. DE, Drazner MH, et al. 2013 ACCF/AHA guideline for the management of heart failure. A report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* 2013;62(16):e147-239. doi:doi: 10.1016/j.jacc.2013.05.019

30. Ziaeian B, Fonarow GC. Epidemiology and aetiology of heart failure. *Nat Rev Cardiol.* Mar 3 2016. doi:10.1038/nrcardio.2016.25

31. Haykowsky MJ, Brubaker PH, Morgan TM, Kritchevsky S, Eggebeen J, Kitzman DW. Impaired aerobic capacity and physical functional performance in older heart failure patients with preserved ejection fraction: role of lean body mass. *J. Gerontol. A. Biol. Sci. Med. Sci.* Aug 2013;68(8):968-975. doi:10.1093/gerona/glt011

32. Hashimoto A, Palmar EL, Scott JA, Abraham SA, Fischman AJ, Force TL, et al. Complications of exercise and pharmacologic stress tests: differences in younger and elderly patients. *J. Nucl. Cardiol.* Nov-Dec 1999;6(6):612-619.

33. Charlson M, Szatrowski TP, Peterson J, Gold J. Validation of a combined comorbidity index. *J. Clin. Epidemiol.* Nov 1994;47(11):1245-1251.

34. Balke B, Ware RW. An experimental study of fitness of Air Force personnel. *U.S. Armed Forces Medical Journal.* 1959;10:678-688.

35. Gibbons RJ, Balady GJ, Bricker JT, Chaitman BR, Fletcher GF, Froelicher VF, et al. ACC/AHA 2002 guideline update for exercise testing: summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1997 Exercise Testing Guidelines). *Circulation.* 2002;106(14):1883-1892.

36. Lipkin DP, Bayliss J, Poole-Wilson PA. The ability of a submaximal exercise test to predict maximal exercise capacity in patients with heart failure. *Eur. Heart J.* Oct 1985;6(10):829-833.

37. Nakajima K, Takeoka M, Mori M, Hashimoto S, Sakurai A, Nose H, et al. Exercise effects on methylation of ASC gene. *Int. J. Sports Med.* 2010;31:671-375. doi:10.1055/s-0029-1246140

38. Arslan S, Erol MK, Gundogdu F, Sevimli S, Aksakal E, Senocak H, et al. Prognostic value of 6-minute walk test in stable outpatients with heart failure. *Tex. Heart Inst. J.* 2007;34(2):166-169.

39. Van Tassell BW, Arena RA, Toldo S, Mezzaroma E, Azam T, Seropian IM, et al. Enhanced interleukin-1 activity contributes to exercise intolerance in patients with systolic heart failure. *PloS one.* 2012;7(3):e33438. doi:10.1371/journal.pone.0033438

40. Yamaoka-Tojo M, Tojo T, Inomata T, Machida Y, Osada K, Izumi T. Circulating levels of interleukin 18 reflect etiologies of heart failure: Th1/TH2 cytokine imbalance exaggerates the pathophysiology of advanced heart failure. *J. Card. Fail.* 2002;8(1):21-27.

41. Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, et al. Evidence for altered interleukin 18 (IL)-18 pathway in human heart failure. *FASEB J.* Nov 2004;18(14):1752-1754. doi:10.1096/fj.04-2426fje

42. Novick D, Kim SH, Fantuzzi G, Reznikov LL, Dinarello CA, Rubinstein M. Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity.* Jan 1999;10(1):127-136.

43. Borlaug BA. The pathophysiology of heart failure with preserved ejection fraction. *Nat Rev Cardiol.* Sep 2014;11(9):507-515. doi:10.1038/nrcardio.2014.83

44. ElGuindy A, Yacoub MH. Heart failure with preserved ejection fraction. *Global cardiology science & practice.* 2012;2012(1):10. doi:10.5339/gcsp.2012.10

45. Luscher TF. Heart failure and left ventricular remodelling in HFrEF and HFpEF. *Eur. Heart J.* Feb 1 2016;37(5):423-424. doi:10.1093/eurheartj/ehw004

46. van Heerebeek L, Paulus WJ. Understanding heart failure with preserved ejection fraction: where are we today? *Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation.* Feb 24 2016. doi:10.1007/s12471-016-0810-1

47. Komajda M, Lam CS. Heart failure with preserved ejection fraction: a clinical dilemma. *Eur. Heart J.* Apr 2014;35(16):1022-1032. doi:10.1093/eurheartj/ehu067

48. Bossaller L, Chiang PI, Schmidt-Lauber C, Ganesan S, Kaiser WJ, Rathinam VA, et al. Cutting edge: FAS (CD95) mediates noncanonical IL-1beta and IL-18 maturation via caspase-8 in an RIP3-independent manner. *J. Immunol.* Dec 15 2012;189(12):5508-5512. doi:10.4049/jimmunol.1202121

49. Toldo S, Mezzaroma E, O'Brien L, Marchetti C, Seropian IM, Voelkel NF, et al. Interleukin-18 mediates interleukin-1-induced cardiac dysfunction. *American journal of physiology. Heart and circulatory physiology.* Apr 1 2014;306(7):H1025-1031. doi:10.1152/ajpheart.00795.2013

50. Stuyt RJ, Netea MG, Verschueren I, Dinarello CA, Kullberg BJ, van der Meer JW. Interleukin-18 does not modulate the acute-phase response. *Journal of endotoxin research.* 2005;11(2):85-88. doi:10.1179/096805105x35170

51. Jitprasertwong P, Jaedicke KM, Nile CJ, Preshaw PM, Taylor JJ. Leptin enhances the secretion of interleukin (IL)-18, but not IL-1beta, from human monocytes via activation of caspase-1. *Cytokine.* Feb 2014;65(2):222-230. doi:10.1016/j.cyto.2013.10.008

52. Horsburgh S, Robson-Ansley P, Adams R, Smith C. Exercise and inflammation-related epigenetic modifications: focus on DNA methylation. *Exerc. Immunol. Rev.* 2015;21:26-41.

53. Kang SJ, Wang S, Hara H, Peterson EP, Namura S, Amin-Hanjani S, et al. Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J. Cell Biol.* May 1 2000;149(3):613-622.

54. Wawrocki S, Druszczynska M, Kowalewicz-Kulbat M, Rudnicka W. Interleukin 18 (IL-18) as a target for immune intervention. *Acta Biochim. Pol.* Feb 17 2016. doi:10.18388/abp.2015_1153

55. Ouzounidis N, Giakoustidis A, Poutahidis T, Angelopoulou K, Iliadis S, Chatzigiagkos A, et al. Interleukin 18 binding protein ameliorates ischemia/reperfusion-induced hepatic injury in mice. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society.* Feb 2016;22(2):237-246. doi:10.1002/lt.24359

56. Gu H, Xie M, Xu L, Zheng X, Yang Y, Lv X. The protective role of interleukin-18 binding protein in a murine model of cardiac ischemia/reperfusion injury. *Transpl. Int.* Dec 2015;28(12):1436-1444. doi:10.1111/tri.12683

57. Negi SI, Pankow JS, Fernstrom K, Hoogeveen RC, Zhu N, Couper D, et al. Racial differences in association of elevated interleukin-18 levels with type 2 diabetes: the Atherosclerosis Risk in Communities study. *Diabetes Care.* Jul 2012;35(7):1513-1518. doi:10.2337/dc11-1957

58. Wang J, Sun C, Gerdes N, Liu C, Liao M, Liu J, et al. Interleukin 18 function in atherosclerosis is mediated by the interleukin 18 receptor and the Na-Cl co-transporter. *Nat. Med.* Jul 2015;21(7):820-826. doi:10.1038/nm.3890

59. Corra U, Mezzani A, Giordano A, Pistono M, Gnemmi M, Caruso R, et al. Peak oxygen consumption and prognosis in heart failure: 14 mL/kg/min is not a "gender-neutral" reference. *Int. J. Cardiol.* Jul 15 2013;167(1):157-161. doi:10.1016/j.ijcard.2011.12.055

60. de Meirelles L, Matsuura C, Resende AD, Salgado ÂA, Pereira NR, Coscarelli PG, et al. Chronic exercise leads to antiaggregant, antioxidant and anti-inflammatory effects in heart failure patients. *European Journal of Preventive Cardiology.* 2013;epub ahead of print.

61. Vaduganathan M, Greene SJ, Butler J, Sabbah HN, Shantsila E, Lip GYH, et al. The immunological axis in heart failure: importance of the leukocyte differential. *Heart Failure Rev* 2013;18(6):835-845. doi:10.1007/s10741-012-9352-9

62. Feiereisen P, Vaillant M, Gilson G, Delagardelle C. Effects of different training modalities on circulating anabolic/catabolic markers in chronic heart failure. *Journal of Cardiopulmonary Rehabilitation and Prevention.* 2013;33:303-308.

63. Smart NA, Steele M. The effect of physical training on systemic proinflammatory cytokine expression in heart failure patients: a systematic review. *Congestive Heart Failure.* 2011;17(3):110-114.

64. Nunes RB, Alves JP, Kessler LP, Lago PD. Aerobic exercsie improves the inflammatory profile correlated with cardiac remodeling and function in chronic heart failure rats. *Clin. Chest Med.* 2013;68(6):876-882.

65. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 2009;27:519-550.

66. O'Connor CM, Whellan DJ, Lee KL, Keteyian SJ, Cooper LS, Ellis SJ, et al. Efficacy and safety of exercise training in patients with chronic heart failure: HF-ACTION randomized controlled trial. *JAMA.* 2009;301(14):1439-1450.

Table 3.1. Demographic and Clinical Characteristics

Characteristic N=54	**Mean ± SD**	**Range**
Age (years)	59.46 ± 9.7	40 – 75
Ejection Fraction (%)	34. ± 14.8	15 – 65
BMI (kg/m^2)	31.37 ± 6.8	19.1 – 49.7
CCI	3.84 ± 1.8	1 –9
	N	**%**
Female	28	51.9%
African American	32	59.3%
HFrEF	34	63.0%
β-Blocker use	46	86.8 %
ACE inhibitor use	23	45.6%
ARB use	13	24.1%
Diuretic use	45	83.3%
Pacer/ICD Device	30	55.6%
College Education	27	50.0%

HFrEF = Heart failure with reduced ejection fraction

BMI = Body mass index

CCI = Charlson Comorbidity Index

ACE = Angiotensin converting enzyme

ARB = Angiotensin receptor blocker

Table 3.2. Physical Measures by Gender (Mean ± SD)

Measure	Total N=54	Females n=28	Males n=26	p-value
Peak $\dot{V}O_2$ (ml/kg/min)	16.68 ± 4.7	14.59 ± 3.6	18.85 ± 4.8	.001
Respiratory Quotient ($\dot{V}CO_2/\dot{V}O_2$)	0.95 ± 0.1	0.89 ± 0.1	1.01 ± 0.1	<.001
Total Treadmill Time (minutes)	8.27 ± 4.4	6.16 ± 3.0	10.58 ± 4.5	<.001
Six Minute Walk Test (meters)	348.9 ± 78.2	338.77 ± 76.7	359.81 ± 79.9	.33

Table 3.3. ASC and Cytokines

Measure	Mean ± SD	Range
ASC Methylation (%)	5.84 ± 0.8	1.33 – 6.82
IL-1β (pg/mL)	1.72 ± 0.8	0 – 5.18
IL-18 (pg/mL)	158.49 ± 56.2	96.2 – 333.1

ASC = apoptosis associated speck-like protein with a caspase recruitment domain

IL = interleukin

Table 3.4. Multivariate Analysis of Predictors of Aerobic Capacity

	Aerobic Capacity (Peak $\dot{V}O_2$ ml/kg/min)		
N=54	Model 1[a]	Model 2[b]	
Variable	β	β	95% CI
Constant	0.44	4.35	[-4.41, 13.10]
% ASC Methylation	2.73**	2.30**	[0.91, 3.70]
LVEF		-0.78*	[-0.15, -0.002]
Gender†		2.42*	[0.07, 4.76]
R^2	.22	.40	
F	12.90**	9.72***	
ΔR^2		.18	
ΔF		6.54**	
	Aerobic Capacity (Peak $\dot{V}O_2$ ml/kg/min)		
N=54	Model 1[c]	Model 2[d]	
Variable	β	β	95% CI
Constant	20.38***	21.51***	[16.73, 26.29]
IL-1β	-2.15**	-1.91**	[-3.32, -0.51]
LVEF		-0.085**	[-0.17, -.004]
Gender†		2.62*	[0.12, 5.12]
R^2	.15	.35	
F	8.47**	8.20***	
ΔR^2		.20	
ΔF		6.99**	

ASC = apoptosis associated speck-like protein containing a caspase recruitment domain.

LVEF = left ventricular ejection fraction. CI = confidence interval.

*p<.05. **p<.01 ***p<.001

[†]Female is coded as 0.

[a]Model 1 is the direct effect of percent ASC Methylation not adjusted for covariates.

[b]Model 2 considered age, gender, six-minute walk test, and BMI covariates for inclusion in the final model in addition to percent ASC Methylation: stepwise variable selection used.

[c]Model 1 is the direct effect of IL-1β not adjusted for covariates.

[d]Model 2 considered age, gender, six-minute walk test, and BMI covariates for inclusion in the final model in addition to IL-1β: stepwise variable selection used.

Chapter IV

Effects of an exercise intervention on ASC methylation and IL-1 cytokines in persons with heart failure

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Abstract

Introduction: Inflammation contributes to heart failure (HF) progression and interleukin (IL)-1 cytokines IL-1β and IL-18 are implicated in this process. The adaptor protein ASC (apoptosis associated speck-like protein containing a caspase recruitment domain) is necessary for inflammasome activation of IL-1β and IL-18. Lower ASC methylation is associated with worse outcomes in HF.

Methods: Participants (N=54) were randomized to receive exercise intervention (n=38) or attention control (n=16) for 3 months. Percent methylation of the ASC gene, plasma IL-1β, and IL-18, and ASC mRNA and were obtained at baseline, 3 months, and 6 months.

Results: ASC methylation was higher in the exercise group as compared to control at 3 months (6.10±0.5% vs. 5.80±0.4%; p=.04) and 6 months (6.07±0.4 vs. 5.82±0.4; p=.04). Plasma IL-1β was lower in the exercise group 3 months (1.43±0.5 pg/mL vs. 2.09±1.3 pg/mL; p=.02) and 6 months (1.49±0.5 pg/mL vs. 2.13±1.4 pg/mL; p=.004). In the exercise group, ASC methylation was higher at 3 months as compared to baseline (p=.009), and IL-1β was lower than baseline at both 3 (p<.001) and 6 months (p=.04). ASC mRNA expression was negatively associated with ASC methylation at baseline (r=-.97, p=.001), 3 months (r=-.90, p=.001), and 6 months (r=-.81, p=.001). ASC mRNA was lower than baseline at 3 months (p=.004) and 6 months (p=.002) among those in the exercise group.

Conclusions: Exercise was related to increased mean percent ASC methylation and decreased IL-1β and ASC mRNA gene expression in HF. Epigenetic regulation of ASC can be a biological mechanism by which exercise can promote better outcomes in HF.

Introduction

Heart failure (HF) is a progressive, terminal illness with frequent decompensation related to ventricular remodeling. Because HF remains a leading cause of morbidity and mortality in the United States,[1,2] identification of novel therapeutic targets that may slow disease progression are urgently needed. HF results from any structural or functional impairment of ventricular filling or ejection of blood and is associated with a chronic sterile inflammation characterized by the formation and activation of a protein complex, the inflammasome, which activates inflammatory cytokines that promote cardiac hypertrophy and myocardial apoptosis.[3-5] The inflammasome is composed of a NOD (nucleotide-binding oligomerization domain)-like receptor (NLRP3), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1.[6-8]

Interleukin-1β (IL-1β) and IL-18 are pleiotropic, inflammatory cytokines theorized to play a prognostic and mechanistic role in HF;[9-13] increased levels have been shown to significantly contribute to worsening HF severity and mortality.[9,14,15] IL-1β and IL-18 increase during acute HF decompensation, suggesting a role in myocardial dysfunction.[14] IL-18 induces the production of tumor necrosis factor-alpha (TNFα),[11] while IL-1β induces nitric oxide production (iNOS),[16] leading to worsening of ventricular remodeling in HF. IL-1β and IL-18 are activated via caspase-1 dependent proteolytic cleavage.[12] Caspase-1 is recruited by an adaptor molecule, ASC, which leads to IL-1β and IL-18 activation.[16] Unlike NLRP3 and caspase-1, ASC has no known independent activity outside of the inflammasome and is necessary for caspse-1

activation.[17] Thus, ASC availability is likely a limiting factor in inflammasome activation.

ASC gene expression is epigenetically controlled by DNA methylation.[18,19] Increased methylation of 7 CpG sites in the promoter region of exon 1 of the ASC gene is associated with decreased ASC mRNA and protein expression.[19] ASC methylation is positively associated with six-minute walk test total distance and aerobic capacity in HF.[19,20] Increased ASC methylation has been shown to be higher in older adults who participated in an aerobic exercise program as compared to controls.[18] However, no studies to date have examined changes in ASC methylation in response to an exercise intervention in persons with HF.

Although no intervention studies targeting epigenetic changes with exercise in HF have been reported to date, studies in healthy adults and adults with chronic diseases, such as breast cancer and diabetes, have demonstrated that short-term and long-term exercise interventions can result in genome-wide and gene-specific epigenetic changes.[21-25] The 2013 American Heart Association recommendations for HF[1] include exercise as a safe and effective non-pharmacological therapy that improves physical and psychological function, reduces hospital readmission rates, lowers mortality in some studies, and improves quality of life.[26-28]

Moderate exercise has been shown to reduce inflammation, and it has been suggested that changes in inflammation with exercise is due to epigenetic changes.[29]

The purpose of this study was to examine the effects of exercise on changes in ASC methylation and activation of the interleukin-1 family cytokines IL-1β and IL-18 in persons with HF. We hypothesized that: 1) persons with HF who participate in a 3-

month aerobic exercise intervention will have increased ASC methylation at completion of the intervention as compared to persons with HF in an attention-control group, and 2) increased ASC methylation will be related to decreased plasma IL-1β, IL-18, and TNFα and to iNOS mRNA expression in persons with HF (Figure 1). Further, we investigated if changes in ASC methylation, plasma IL-1β, IL-18, and TNFα were sustained 3 months after completing the aerobic exercise intervention.

Methods

Study Design

This study was an exploratory sub-study of a 3-year randomized controlled intervention feasibility study, as previously described.[30] Fifty-four participants were enrolled from 1 of 4 large urban tertiary-care hospitals that had multidisciplinary outpatient HF clinics. Participants were randomized to receive an exercise intervention (n=38) or attention control (n=16) for 3 months. Participants were followed for an additional 3-month maintenance phase after the intervention. Both groups received two home baseline visits, weekly phone calls during the 3-month intervention phase, and bi-monthly phone calls during the maintenance phase, from 3 months to 6 months. The time points were chosen to examine physiological changes over a short-term (3 month) moderate intensity exercise program and if the changes persisted or diminished 3 months post exercise intervention (at 6 months).

The exercise group received the exercise prescription using a progressive, moderate intensity aerobic protocol, as previously described.[30] To ensure that participants achieve adequate training stimulus, dose-specific exercise was based on maximum heart

rate obtained during symptom-limited, modified Balke treadmill test at baseline. Participants in the exercise group were instructed to walk for 30 minutes 3 times per week for the first two weeks followed by 45 minutes 3 times per week for the remaining 10 weeks. The attention control group received education and flexibility and stretching exercises to control for the possible confounding variable of receiving attention from a healthcare professional.[30]

All studies were performed under research protocols approved by the Institutional Review Boards of Emory University and participating institutions. Each subject was informed of testing protocols and the potential risks and benefits of participation. All participants provided written consent before participation.

Study Sample

Participants were screened for eligibility via medical record. Inclusion criteria were: documented medical diagnosis of NYHA class II or III, aged 40-75 years; left ventricular ejection fraction (LVEF) ≥10% documented within the last year by echocardiogram, cardiac catheterization ventriculography, or radionuclide ventriculography; and receiving medication therapy for HF according to the American College of Cardiology/American Heart Association recommendation guidelines for at least 8 weeks prior to study enrollment. Exclusion criteria were: medical diagnosis of NYHA class I or IV; change in HF therapy within the previous 8 weeks; worsening HF symptoms within the last 5 days; unstable angina; renal insufficiency (serum creatinine > 3.0 mg/dL); fixed rate pacemaker; uncontrolled hypertension, involved in any structured exercise program or exercising 3 or more times per week for a minimum of 30 minutes;

hospitalization within the previous 30 days; and any disorder precluding an exercise treadmill test.[30]

Severity of illness was controlled by limiting participants to NYHA class II and III, who are more similar in response to exercise than class I and IV. In addition, severity of illness was controlled by LVEF limitations (LVEF > 10%) and optimal medication therapy for HF. Participants below the age of 40 years are likely to have HF for other reasons than the majority of the general HF population. Older age (>75) is associated with reduced exercise capacity which may confound the physiological outcome measurements. Both resting and exercise heart rates are influenced by beta-blockers, which is considered optimal therapy for HF patients, and this was controlled for during analysis. For both exercise testing and training, the heart rate reserve method was used, which takes into account the patient's resting heart rate, thereby reducing the effect of beta-blockade. Best efforts were made to schedule patients for their exercise testing and training a minimum of three hours after taking beta blockers. Patients with an ICD were enrolled if their heart rate limits were set to be higher than the target heart rate for the exercise regimen. Participants with recurrent angina, more severe symptoms, or have uncontrolled hypertension were excluded due to the higher risk for adverse cardiovascular events during exercise testing and the walking intervention. Because the benefit of exercise is being evaluated, participants who were currently or recently enrolled in an exercise program for the previous eight weeks or were exercising at regular intervals (more than twice per week for 30 minutes) were excluded from the study.

G*Power software was used to assess the power/effect size detectable given an estimated sample size of 54. A sample size of 54 at 80% power can detect large effect

sizes in the repeated measures analysis (f=0.37 for group main effect, f=0.41 for time main effect and f=0.41 for the group-x-time interaction effect) using an F-Test with a significance level (alpha) of 0.05.

Measurements

Demographic and Clinical Data

Sociodemographic and clinical variables included age, gender, medical history, and medications, and were obtained from medical records and a self-report questionnaire. The Charlson Comorbidity Index (CCI)[31] was used to assess for other chronic conditions. Height was measured with a standard stadiometer, without shoes and recorded in centimeters. Weight was measured in kilograms using a calibrated scale with the participant in light clothing, without shoes. Body mass index (BMI) was calculated by the formula: BMI = (weight in kg)/(height in cm)^2. Participants with LVEF <40% were categorized as heart failure with reduced ejection fraction (HFrEF), and those with LVEF ≥40% were categorized as preserved ejection fraction (HFpEF).

Blood draws took place at BL, intervention completion (3 months) and after a 3-month maintenance period (6 months). Blood samples were collected in the morning after an overnight fast. Blood was collected in a vacutainer with EDTA, separated into plasma and buffy coat, and stored at -80°C until analysis.

Exercise Logs

Participants were provided 3 calendars to record exercise sessions completed during each month of the intervention. Participants who documented at least 12 exercise

sessions per month were considered to have completed the exercise program. Participants who recorded less than 12 exercise sessions per month or did not turn in any completed exercise calendars were considered to have not completed the exercise program.

Cardiopulmonary Exercise Stress Test

The dose-specific exercise was based on maximum heart rate obtained during a modified Balke maximal symptom-limited treadmill test.[32,33] The test protocol and parameters have been described previously.[30] In brief, heart rate, continuous gas exchange, telemetry, blood pressure, rating of perceived exertion, and oxygen saturation were assessed for each patient 1 minute before, during, and 4 minutes after the exercise test according to American Heart Association guidelines.[34] The test protocol began at 0% incline at 2.0 mph on a motorized treadmill for 3 minutes with an increase in incline of 3.5% every 3 minutes for 18 minutes. All participants completed the treadmill test regardless of group assignment.

ASC Methylation

Percent methylation of 7 CpG sites in the intron region of ASC was measured as previously reported.[19] In brief, genomic DNA from peripheral blood mononuclear cells (PBMCs) was bisulfite treated and amplified by PCR followed by pyrosequencing for methylation quantification.[18] Methylation of 7 CpG sites in the promoter region of exon 1 were measured[19] and analyzed as mean percent methylation. The mean percent

methylation of the 7 CpG sites for each individual was calculated as the sum of percent methylation of the CpG sites divided by 7.

Cytokines

IL-1β, IL-18, and TNFα were analyzed from plasma that has been separated from collected whole blood and stored at -80°C immediately after collection. Plasma cytokines were measured in duplicate using commercially available ELISA kits (eBioscience). Plates were read on a BioTek microplate reader and analyzed using Gen5 software. Curve fitting was selected among linear, quadratic and 4-point based on the best regression coefficient.

mRNA

mRNA was extracted using a commercial kit (mRNA Catcher^{TM} Plus, Invitrogen) and converted to cDNA using reverse transcriptase PCR (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). IL-18, ASC, and iNOS mRNA were quantified via quantitative real-time PCR (RT-PCR). GAPDH was used as the reference gene. Primers used for RT-PCR were developed using Primer Express® software and are listed in Table 1. To normalize gene expression relative to a non-HF reference sample, mRNA quantification results were calculated using the $2^{-\Delta\Delta CT}$ method.[35]

Data Analysis

Descriptive statistics were analyzed for all study variables and data were reviewed for normality assumptions and outliers, in preparation for analysis. IL-18 did not met

criteria for normality and was log transformed (LN) for statistical analysis. All data were analyzed using SAS version 9.4 with an alpha set at 0.05. Student t-tests were used to compare group differences in demographic and clinical variables at baseline. PROC MIXED with Bonferroni adjusted least squares mean analysis was used to compare within GROUP and between GROUP changes across TIME. For hypothesis testing, data was analyzed according to intention to treat principles. The primary analysis used to test the hypotheses employed a general linear mixed model for repeated measures data. For most variables, the model has one between-subjects variable GROUP with two levels (*control and exercise*) and one within-subjects variable TIME with three levels *(BL, 3 and 6 months***).** The test of the hypotheses hinged on the GROUP by TIME interaction as an indication of group differences in the variable of interest over time. A given hypothesis was supported by the finding of a statistically significant GROUP by TIME interaction, *and* the finding that the means are in the hypothesized direction. Multilevel modeling, using PROC MIXED, provided separate estimates of the means for each variable by time and treatment groups. This method allowed to control for attrition over time. The linear model was fit using restricted maximum likelihood estimation with an appropriate form for the variance-covariance among the repeated measures. Covariates for each hypothesis were selected based on literature documentation of relationships and performance of the data and relationships within this study. PROC MIXED allowed for the handling of missing data through the use of restricted maximum likelihood estimation, retaining participants in the analysis and preserving sample size, and accommodated covariates as defined in the model. Effect sizes were calculated using Hedges' g, which adjusts the calculation of the pooled standard deviation with weights

for the sample sizes.[36] Mediation models to examine the indirect effects of ASC methylation on downstream variables via IL-1 cytokines were performed using PROC IML with PROCESS version 2.15.[37]

Results

Patient Characteristics

Demographic and clinical data are presented in Table 2. No significant group differences at baseline were found. Age varied widely, ranging from 40 to 75 years. LVEF ranged from 15 to 65%, and 65.0% of the participants had LVEF <40%. The most common comorbidities were hypertension (N=36, 66.7%), dyslipidemia (N=30, 55.6%), depression (N=20, 37.0%), and diabetes (N=19, 35.2%). One-third of participants (N=16, 29.6%) had a previous MI. Older participants had higher LVEF (r=.281, p=.044), more comorbidities (CCI, r=.75, p<.001), and had lower total distance walked on 6MWT (r=-.463, p<.001). Females had higher LVEF as compared to males (38.23 ± 14.9 vs. 29.44 ± 13.6, respectively; t=2.26, p=.03).

ASC Methylation and Cytokines by Group and over Time

ASC methylation and cytokine data are presented by group and time in Table 3. No significant group differences at baseline were found. Mean percent ASC methylation was higher in the exercise group as compared to the control group at 3 months and 6 months, with medium to large effects sizes of .72 and .81[36], respectively (Table 3). Plasma IL-1β was lower among the exercise group as compared to the control group at 3 months and 6 months, with medium to large effect sizes of .83 and .79[36], respectively

(Table 3). Among those in the exercise group mean percent ASC methylation was higher at 3 months as compared to baseline, and IL-1β was significantly lower than baseline at both 3 months and 6 months (Table 3). Significant group differences in change scores from baseline to 3 months (Figure 2) were found for IL-1β (control 0.18 pg/mL vs exercise -0.33 pg/mL, t=3.73, p=.001) and mean percent ASC methylation (control -0.04% vs exercise 0.04%, t=-2.71, p=.01). In addition, group differences in change scores from baseline to 6 months (Figure 2) were found for IL-1β (control 0.19 pg/mL vs exercise -0.62 pg/mL, t=2.65, p=.013) and TNFα (control 0.23 pg/mL vs exercise -0.22 pg/mL, t=2.79, p=.01).

Mean percent ASC methylation was negatively associated with IL-1β at baseline (r=-.443, p=.001), 3 months (r=-.533, p=.01), and 6 months (r=-.477, p=.009). IL-18 was positively associated with TNFα at 3 months (r=.467, p=.005), but not at baseline or 6 months. IL-1β change scores were positively associated with changes in IL-18 from baseline to 3 months (r=.422, p=.020) and from 3 months to 6 months (r=.411, p=.037). Further, IL-1β change scores were positively associated with changes in TNFα from baseline to 3 months (r=.609, p=.001) and from baseline to 6 months (r=.395, p=.034).

IL-18, ASC, and iNOS mRNA

There were no significant intra-individual changes over time or inter-individual group differences over time in IL-18 mRNA (Figure 3). There were significant differences in ASC mRNA expression between control group and exercise group (Figure 3) at 3 months (14.95 ± 2.5 vs. 8.46 ± 1.3, respectively; t=2.27; p=.027) and at 6 months (11.60 ± 3.1 vs. 6.32 ± 1.0, respectively; t=2.24, p=.29), but not at baseline (10.87 ± 2.2

vs. 9.00 ± 2.0, respectively; t=0.55, p=.58). For the exercise group, there were within-group changes in ASC mRNA from baseline to 3 months (t=-3.01; p=.004), from baseline to 6 months (t=-3.21; p=.002), and from 3 months to 6 months (t=4.82; p=.001). There were significant group differences in iNOS mRNA expression between control group at exercise group at 6 months (2.78 ± 0.2 vs. 1.72 ± 0.2; t=3.51, p=.001), but not at baseline or 3 months (Figure 3). Within the control group, there was a significant drop in iNOS mRNA expression from baseline to 3 months (t=2.33, p=.02), but the values returned to baseline levels at 6 months (baseline to 6 months: t=-0.45, p=.65; 3 months to 6 months: t=-0.31, p=.003). There were significant within-group iNOS mRNA changes for the exercise group from baseline to 3 months (t=3.32, p=.002), which remained significant at 6 months (t=2.65, p=.011).

ASC mRNA was negatively associated with mean percent ASC methylation at baseline (r=-.97, p=.001), 3 months (r=-.90, p=.01), and 6 months (r=-.89, p=.001) and was positively associated with plasma IL-1β levels at baseline (r=.39, p=.003), 3 months (r=.48, p=.004), and 6 months (r=.42, p=.02). iNOS mRNA was positively related to plasma IL-1β levels at baseline (r=.72, p=.001) and 6 months (r=.84, p=.001) and was negatively associated with mean percent ASC methylation at baseline (r=-.34, p=.011) and 6 months (r=-.45, p=.015). iNOS mRNA was positively associated with plasma TNFα levels at 6 months (r=.49, p=.007). We hypothesized that increased ASC methylation would be related to decreased iNOS expression caused by a decrease in IL-1β. Due to the multicollinearity between ASC methylation, iNOS mRNA, and IL-1β, we tested a mediation model in which IL-1β mediates the relationship between ASC methylation and iNOS mRNA expression. There was a significant indirect effect of ASC

methylation on iNOS mRNA expression via IL-1β at baseline (effect -0.62, 95% CI [-1.15, -0.41]) and 6 months (effect -0.23, 95% CI [-0.80, -0.04].

Multilevel Models for Change

To examine factors related to ASC and cytokine levels and to factors related to rate of change over time, clinical and demographic variables theorized to be related to inflammation were used as predictors in the multilevel model for change (Table 4). Models were created for the combined sample to examine effects of the exercise intervention and for the exercise only group to examine factors related to changes over time within the exercise intervention.

Combined Sample

Mean percent ASC methylation at baseline was positively associated with LVEF and the interaction effect of peak $\dot{V}O_2$ and gender. Group assignment was associated with rate of change over time such that participants in the intervention group had a higher rate of change than the control group (Table 4). Baseline plasma IL-1β levels were negatively associated with LVEF and the interaction effect of peak $\dot{V}O_2$ and gender. While group assignment to the exercise group was associated with a negative rate of change in IL-1β (β=-0.11), the rate of change was higher among those who reported they completed the prescribed exercise program (β=-0.6). Although peak $\dot{V}O_2$ was positively associated with mean percent ASC methylation (r=.47, p=.001) and negatively associated with IL-1β (r=-.38, p=.007) at baseline, the interaction of peak $\dot{V}O_2$ and gender was used in the models due to the significant differences in peak $\dot{V}O_2$ between males and females

(18.85 ± 4.8 vs 14.59 ± 3.6, respectively; t=-3.54, p=.001). No group by time effects using multilevel modeling were found for IL-18, however significant fixed effects were found for race (β=46.34, SE=13.8, p=.003) and BMI (β=2.48, SE=1.0, p=.02). No significant models for TNFα were found.

Exercise Group

In the exercise group analysis, mean percent ASC methylation at baseline remained positively associated with LVEF and the interaction effect of peak $\dot{V}O_2$ and gender (Table 5). Rate of change for participants in the exercise intervention was associated with gender, race, and level of education. Male gender (β=0.04) and having attended college (β=0.04) were associated with a higher rate of change in ASC methylation. The rate of change in ASC methylation was lower for those who identified as non-black (β=-0.06). The exercise group analysis for IL-1β produced results that were similar to the whole group analysis (Table 5). LVEF and the interaction effect of peak $\dot{V}O_2$ and gender remained negatively associated with IL-1β at baseline. Within in the exercise group, those who reported they had completed the exercise program had a negative rate of change (β=-0.06)

Discussion

This is the first study, to our knowledge, to examine the effects of an exercise intervention on epigenetic changes in persons with HF. We hypothesized that persons with HF who participated in a 3-month aerobic exercise intervention would have increased ASC methylation at the completion of the intervention as compared to persons

with HF in an attention-control group. A 3-month exercise intervention in persons with HF was associated with increased ASC methylation and decreased IL-1β at 3 months with medium to large effect sizes, supporting this hypothesis. The increase in ASC methylation after exercise was associated with a decrease in ASC mRNA expression, suggesting that exercise may decrease inflammation in HF via epigenetic control of inflammasome formation due to decreased bioavailability of ASC.

A meta-analysis of changes in DNA methylation associated with exercise found that effect size of DNA methylation change across 16 different publications and 1580 people was large, with a Cohen's d of 1.20.[38] Further, analyses indicated that the effect size of DNA methylation change with exercise was greater for participants over 40 years of age as compared to participants under 40 years, particularly with regard to studies measuring increases in DNA methylation.[38] Genome-wide DNA methylation has been shown to decrease with age,[39-42] and thus, the epigenetic protective effects of exercise are likely more apparent in the face of the age-related changes in DNA methylation of older adults as compared to the younger population. In this study, we only included participants who were at least 40 years of age, and our hypothesis was to find an increase in DNA methylation of ASC after the exercise intervention. Combined with the previous evidence demonstrating higher ASC methylation among older adults (≥ 40 years of age) who exercised as compared to a control group,[18] the medium to large effect sizes related to changes in DNA methylation in this study were not surprising.

Secondly, we hypothesized that increased ASC methylation would be related to decreased plasma IL-1β, IL-18, and TNFα and to iNOS mRNA expression in persons with HF. We also investigated if changes in ASC methylation, plasma IL-1β, IL-18, and

TNFα were sustained 3 months after completing the aerobic exercise intervention. Increased ASC methylation was related to decreased IL-1β at baseline, 3 months, and 6 months but not to IL-18 or TNFα. ASC methylation was also negatively related to iNOS mRNA expression over time, and this relationship was mediated by IL-1β. Changes in IL-1β over time were related to changes in IL-18 and TNFα from baseline to 3 months and from baseline to 6 months. These data suggest that the reduction in inflammatory cytokines after an exercise intervention occur via decreased activation of IL-1β and that this reduction in IL-1β is related to epigenetic control of ASC production.

IL-1β is one of the most important and potent inflammatory mediators in the acute phase response and in the pathophysiology of chronic diseases, such as heart failure;[29,43,44] activation of IL-1β is tightly regulated by the inflammasome.[3,29] IL-1β stimulates nitric oxide production, as evidenced by a corresponding increase in iNOS, leading to further activation of inflammatory mediators.[5,43] IL-1β is also associated with patient report of sickness symptoms,[45,46] perhaps contributing to the common symptoms of fatigue and depression also associated with HF, and, in other studies, reduced by exercise.[47,48] Combined with the associated changes in ASC methylation and expression, these results suggest that exercise decreases inflammation, at least in part, via decreased inflammasome formation.

We expected to see between group and within group changes in IL-18 over time, but none were found. The few studies examining changes in IL-18 after an exercise intervention have had conflicting results, where some studies demonstrated a decrease in IL-18[49,50] and other studies[51-53] did not. There have been no studies to date examining changes in IL-18 after an exercise intervention in persons with HF. While the values of

IL-18 and IL-1β were not associated at any time point, changes in these two cytokines from baseline to 3 months and from 3 months to 6 months were related.

IL-1β is produced as a precursor protein in response to an inflammatory stimulus and inflammasome activation.[8,54-56] IL-18 is constitutively expressed as a biologically inactive precursor molecule that lacks a signal peptide.[57,58] IL-18 mRNA has a long half-life, thus contributing to steady state production of IL-18.[16] Although both IL-1β and IL-18 require caspase-1 dependent proteolytic cleavage for activation,[10,59] IL-1β is more tightly regulated by the inflammasome than IL-18. Additionally, IL-18 is regulated by an endogenous inhibitor, IL-18 binding protein (IL-18BP), which inactivates IL-18 when bound,[60] and thus has a more complex mechanism of function than IL-1β. There is some evidence that IL-1β can also induce IL-18,[58,61] while other studies have shown that IL-18 induces IL-1β.[10,16] The changes in these two cytokines over time suggest that they are changing together even if the actual levels are not related at time points. Further studies are needed to untangle the relationships between these IL-1 family cytokines.

Like IL-18, changes in TNFα were related to changes in IL-1β. This relationship in cytokine change may be due to a decrease in IL-1β activation of IL-18 or just coincide with overall changes IL-1 cytokines related to changes in ASC methylation-driven decrease in inflammasome formation. Group differences in TNFα were only found at 6 months. However, there were significant group differences in the change scores at both 3 months and 6 months, indicating a significant decrease within the exercise group. While some studies examining the effects of an exercise intervention on changes in TNFα in HF have reported decreased TNFα post-exercise intervention,[62-64] others found no significant changes in TNFα after an exercise intervention.[65-67] A systematic review of persons with

HF enrolled in randomized controlled trials with an exercise intervention found that of the 11 studies measuring TNFα before and after the exercise intervention, only 4 studies demonstrated a significant decrease in TNFα.[68] Similar to this study, a trial of 45 persons with HF found within-group changes in TNFα after the exercise intervention, but no group differences were found over time.[62] A sub-analysis within one study that found no overall differences in TNFα after exercise but demonstrated between group changes only in the sub-group of participants with idiopathic dilated cardiomyopathy,[65] suggesting that the etiology of HF may play a role in dynamic changes in TNFα. Further examination of the effects of exercise on TNFα and its effects in persons with HF is needed to better assess how TNFα changes within the heterogeneous HF population.

Our second hypothesis was partially supported in that increased ASC methylation was associated with decreased IL-1β and iNOS, but not with IL-18 and TNFα. Inflammation in HF is initiated by danger-associated molecular patterns (DAMP), which are host-derived molecules indicative of cellular damage.[4,69] It is possible that the effects of exercise led to decreased DAMP formation, the impetus for inflammasome formation and activation, independent of changes in ASC methylation. However, the associations between ASC methylation, ASC mRNA expression, and IL-1β before and after the exercise intervention suggest the changes in ASC methylation likely decrease inflammasome formation and function, at least, to some extent.

No changes over time in IL-18 mRNA expression were found. IL-18 is most abundant in a constitutively expression form,[10] and these results are likely reflective the steady-state levels of IL-18 mRNA in peripheral blood mononuclear cells. There were significant changes in ASC mRNA expression over time. ASC mRNA levels were

negatively associated with ASC methylation at each time point. While we cannot determine causation using this model, this evidence, in addition to our findings in a study with a different HF population, is suggestive of epigenetic control of ASC gene expression in persons with HF.[19] Further mechanistic work is warranted.

In our multilevel modeling analysis, baseline ASC methylation and IL-1β levels were related to LVEF, gender, and peak $\dot{V}O^2$, which we previously reported.[20] The analysis of the entire sample again demonstrated that the exercise intervention was effective in increasing mean percent ASC methylation and in decreasing plasma IL-1β. Response to the exercise intervention was examined by analyzing factors related to rate of change within the exercise group only. We found that males who participated in the exercise intervention had a greater increase in ASC methylation. One study examining DNA methylation and gene expression in skeletal muscle of healthy persons after an exercise intervention found differential DNA methylation patterns between males and females,[70] however no specific patterns or meaningful changes were revealed. No other studies examining gender differences in DNA methylation changes in response to exercise have been reported to date. In this study, the gender difference in the DNA methylation response to the exercise intervention could be related to gender differences in baseline aerobic capacity and body composition. Male participants may have exercised at higher intensities due to higher aerobic capacity or may have had a faster physiological response to exercise due to a higher proportion of muscle mass than female participants. Education could be a proxy measure for socioeconomic status and may be reflective of better access to care, a gym, or reliable exercise space. Income data were not collected, so we were unable to compare the measures for analysis. The IL-1β rate of change was

related to self-reported adherence to the exercise program. This relationship suggests that the amount of exercise is related to the level of decrease in IL-1β in HF, and further suggests that exercise may modulate inflammasome formation or activation. The exercise adherence measure was self-report, and we assumed in our analysis that those who did not submit the exercise calendar did not complete the exercise. Thus, the analysis may not have accurately captured the true level of adherence.

These results demonstrate that behavior can influence gene expression of inflammation via epigenetic regulation of a key inflammatory protein in persons with HF. We followed participants for an additional 3 months after the exercise intervention. Some participants may have continued the walking program, although this was not monitored. If they did, this could have contributed to the prolonged effect. A longer intervention time with longer follow-up may demonstrate the effects of long-term behavior change. Previous studies have demonstrated beneficial effects with walking exercises in persons with HF,[28,63] and here we support the evidence that walking is an appropriate and beneficial level of activity in HF. The possibility that exercise may be able to reverse epigenetic-induced changes in gene expression of inflammatory proteins and other markers associated with HF pathophysiology, thereby improving outcomes, is an area of research greatly in need of further exploration.

Limitations

This study used a short-term exercise program (12 weeks); longer interventions may provide more insight into the dynamics of changes in inflammatory proteins over time and examine the limits and rate of change of DNA methylation with exercise. We

did not capture the level of exercise during the 3-month maintenance phase, so we were unable to identify if changes are sustained at 3 months were due to continued exercise or if there are short term lasting epigenetic and anti-inflammatory effects. Exercise adherence was self-report, and some participants did not provide a self-report exercise log, which we inferred to mean they did not adhere to the protocol as described. Thus, we may have over or underestimated actual adherence. Real-time activity trackers could provide more objective data to better identify the relationships between exercise intensity and endurance with changes in DNA methylation and cytokine expression. The results of this study are specific to the 3-month walking exercise program and may not be generalizable to all types and durations of exercise.

This study measured DNA methylation and mRNA expression in peripheral blood mononuclear cells (PBMCs) and circulating cytokines in plasma. We did not measure changes in other tissues. There may be meaningful tissue-specific epigenetic changes with exercise resulting in altered inflammatory profile in the myocardium and skeletal muscle, and these changes may more tightly align with changes in cytokine expression. We used PBMCs collected from whole blood and did not control for leukocyte differential. Intra-individual and inter-individual differences over time may be related to different leukocyte composition that we were unable to capture.

Conclusions

We demonstrated that an exercise intervention in persons with HF is associated with changes in DNA methylation of a key component of the inflammasome, ASC, and that these changes are associated with decreased ASC gene expression. Further, changes

in ASC methylation and expression were associated with decreased plasma IL-1β among participants in the exercise intervention. Epigenetic regulation of ASC may be a biological mechanism by which exercise can promote better outcomes in HF. Further research examining mechanisms of change can lead to improved understanding of physiological adaptations and more precise prediction of adverse outcomes in persons with HF.

References

1. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* Oct 15 2013;62(16):e147-239. doi:10.1016/j.jacc.2013.05.019
2. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics - 2013 update: A report from the American Heart Association. *Circulation.* 2013;127:e6-e245.
3. Abbate A. The heart on fire: Inflammasome and cardiomyopathy. *Exp. Physiol.* 2013;98(2):385. doi:10.1113/expphysiol.2012.069021
4. Bracey NA, Beck PL, Muruve DA, Hirota SA, Guo J, Jabagi H, et al. The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1β. *Exp. Physiol.* 2013;98(2):462-472. doi:10.1113/expphysiol.2012.068338
5. Pomerantz BJ, Reznikov LL, Harken AH, Dinarello CA. Inhibition of caspase 1 reduces human myocardial ischemic dysfunction via inhibition of IL-18 and IL-1β. *Proc. Natl. Acad. Sci. U. S. A.* 2001;98(5):2871-2876.
6. Schroder K, Zhou R, Tschopp J. The NLRP3 inflammasome: A sensor for metabolic danger? *Science.* 2010;327:296-300. doi:10.1126/science.1184003

7. Tschopp J, Schroder K. NLRP3 inflammasome activation: the convergence of multiple signalling pathwayson ROS production? *Nature Reviews Immunology.* 2010;10:210-215. doi:10.1038/nri2725

8. Schroder K, Tschopp J. The inflammasome. *Cell Adhes. Commun.* 2010;140(821-832). doi:10.1016/j.cell.2010.01.040

9. Eslick GD, Thampan BV, Nalos M, McLean AS, Sluyter R. Circulating interleukin-18 concentrations and a loss-of-function P2x7 polymorphism in heart failure. *Int. J. Cardiol.* 2009;137(1):81-83. doi:10.1016/j.ijcard.2008.05.017

10. Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, et al. Evidence for altered interleukin 18 (IL)-18 pathway in human heart failure. *FASEB J.* Nov 2004;18(14):1752-1754. doi:10.1096/fj.04-2426fje

11. Naito Y, Tsujino T, Fujioka Y, Ohyanagi M, Okamura H, Iwasaki T. Increased circulating interleukin-18 in patients with congestive heart failure. *Heart & lung : the journal of critical care.* 2002;88:296-297.

12. Okamura H, Tsutsui H, Kashiwamura S-I, Yoshimoto T, Nakanishi K. Interleukin-18: A novel cytokinethat augments both innate and acquired immunity. *Adv. Immunol.* 1998;70:281-312.

13. Ouzounidis N, Giakoustidis A, Poutahidis T, Angelopoulou K, Iliadis S, Chatzigiagkos A, et al. Interleukin 18 binding protein ameliorates ischemia/reperfusion-induced hepatic injury in mice. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society.* Feb 2016;22(2):237-246. doi:10.1002/lt.24359

14. Yamaoka-Tojo M, Tojo T, Inomata T, Machida Y, Osada K, Izumi T. Circulating levels of interleukin 18 reflect etiologies of heart failure: Th1/TH2 cytokine imbalance exaggerates the pathophysiology of advanced heart failure. *J. Card. Fail.* 2002;8(1):21-27.

15. Hedayat M, Mahmoudi MJ, Rose NR, Rezaei N. Proinflammatory cytokines in heart failure: double-edged swords. *Heart Failure Reviews.* 2010;15:543-562.

16. Lebel-Binay S, Berger A, Zinzindohoué F, Cugnenc P-H, Thiounn N, Fridman WH, et al. Interleukin-18: Biological properties and clinical implications. *Eur. Cytokine Netw.* 2000;11(1):15-26.

17. Taniguchi S, Sagara J. Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC. *Seminars in Immunopathology.* 2007;29:231-238.

18. Nakajima K, Takeoka M, Mori M, Hashimoto S, Sakurai A, Nose H, et al. Exercise effects on methylation of ASC gene. *Int. J. Sports Med.* 2010;31:671-375. doi:10.1055/s-0029-1246140

19. Butts B, Gary RA, Dunbar SB, Butler J. Methylation of Apoptosis-Associated Speck-Like Protein With a Caspase Recruitment Domain and Outcomes in Heart Failure. *J. Card. Fail.* Dec 14 2015. doi:10.1016/j.cardfail.2015.12.004

20. Butts B, Butler J, Dunbar SB, Corwin EJ, Gary RA. ASC methylation and interleukin-1β are associated with aerobic capacity in heart failure. *UNDER REVIEW.* 2016.

21. Barres R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, et al. Acute exercise remodels promoter methylation in human skeletal muscle. *Cell metabolism.* Mar 7 2012;15(3):405-411. doi:10.1016/j.cmet.2012.01.001

22. Denham J, O'Brien BJ, Marques FZ, Charchar FJ. Changes in the leukocyte methylome and its effect on cardiovascular-related genes after exercise. *Journal of applied physiology (Bethesda, Md. : 1985).* Feb 15 2015;118(4):475-488. doi:10.1152/japplphysiol.00878.2014

23. Ronn T, Volkov P, Davegardh C, Dayeh T, Hall E, Olsson AH, et al. A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. *PLoS genetics.* Jun 2013;9(6):e1003572. doi:10.1371/journal.pgen.1003572

24. Rowlands DS, Page RA, Sukala WR, Giri M, Ghimbovschi SD, Hayat I, et al. Multi-omic integrated networks connect DNA methylation and miRNA with skeletal muscle plasticity to chronic exercise in Type 2 diabetic obesity. *Physiological genomics.* Oct 15 2014;46(20):747-765. doi:10.1152/physiolgenomics.00024.2014

25. Zeng H, Irwin ML, Lu L, Risch H, Mayne S, Mu L, et al. Physical activity and breast cancer survival: an epigenetic link through reduced methylation of a tumor suppressor gene L3MBTL1. *Breast Cancer Res. Treat.* May 2012;133(1):127-135. doi:10.1007/s10549-011-1716-7

26. de Meirelles LR, Matsuura C, Resende Ade C, Salgado AA, Pereira NR, Coscarelli PG, et al. Chronic exercise leads to antiaggregant, antioxidant and anti-

inflammatory effects in heart failure patients. *Eur J Prev Cardiol.* Oct 2014;21(10):1225-1232. doi:10.1177/2047487313491662

27. DeMaeyer C, Beckers P, Vrints CJ, Conraads VM. Exercise training in chronic heart failure. *Therapeutic Advances in Chronic Disease.* 2013;4(3):105-117.
28. O'Connor CM, Whellan DJ, Lee KL, Keteyian SJ, Cooper LS, Ellis SJ, et al. Efficacy and safety of exercise training in patients with chronic heart failure: HF-ACTION randomized controlled trial. *JAMA.* Apr 8 2009;301(14):1439-1450. doi:10.1001/jama.2009.454
29. Horsburgh S, Robson-Ansley P, Adams R, Smith C. Exercise and inflammation-related epigenetic modifications: focus on DNA methylation. *Exerc. Immunol. Rev.* 2015;21:26-41.
30. Gary RA. Intervention Effects Paper. 2016.
31. Charlson M, Szatrowski TP, Peterson J, Gold J. Validation of a combined comorbidity index. *J. Clin. Epidemiol.* Nov 1994;47(11):1245-1251.
32. Balke B, Ware RW. An experimental study of fitness of Air Force personnel. *U.S. Armed Forces Medical Journal.* 1959;10:678-688.
33. Gibbons RJ, Balady GJ, Bricker JT, Chaitman BR, Fletcher GF, Froelicher VF, et al. ACC/AHA 2002 guideline update for exercise testing: summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1997 Exercise Testing Guidelines). *Circulation.* 2002;106(14):1883-1892.

34. Balady GJ, Arena R, Sietsema K, Myers J, Coke L, Fletcher GF, et al. Clinician's Guide to cardiopulmonary exercise testing in adults: a scientific statement from the American Heart Association. *Circulation.* 2010;122:191-225.

35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* Dec 2001;25(4):402-408. doi:10.1006/meth.2001.1262

36. Hedges LV. Distribution Theory for Glass's Estimator of Effect size and Related Estimators. *Journal of Educational and Behavioral Statistics.* June 20, 1981 1981;6(2):107-128. doi:10.3102/10769986006002107

37. Hayes AF. *Introduction to mediation, moderation, and conditional process analysis : a regression-based approach.* New York: New York : The Guilford Press; 2013.

38. Brown WM. Exercise-associated DNA methylation change in skeletal muscle and the importance of imprinted genes: a bioinformatics meta-analysis. *Br. J. Sports Med.* Dec 2015;49(24):1567-1578. doi:10.1136/bjsports-2014-094073

39. Zinovkina LA, Zinovkin RA. DNA Methylation, Mitochondria, and Programmed Aging. *Biochemistry (Mosc).* Dec 2015;80(12):1571-1577. doi:10.1134/s0006297915120044

40. Smith JA, Zagel AL, Sun YV, Dolinoy DC, Bielak LF, Peyser PA, et al. Epigenomic Indicators of Age in African Americans. *Hereditary genetics : current research.* Dec 2014;3(3). doi:10.4172/2161-1041.1000137

41. Ono T, Uehara Y, Kurishita A, Tawa R, Sakurai H. Biological significance of DNA methylation in the ageing process. *Age Ageing.* Jan 1993;22(1):S34-43.

42. Mays-Hoopes LL. DNA methylation in aging and cancer. *J. Gerontol.* Nov 1989;44(6):35-36.

43. van Tassell B, Arena RA, Toldo S, Mezzaroma E, Azam T, Seropian IM, et al. Enhanced interleukin-1 activity contributes to exercise intolerance in patients with systolic heart failure. *PLoS ONE.* 2012;7(3):e33438. doi:10.1371/journal.pone.0033438

44. Kawaguchi M, Takahashi M, Hata T, Kashima Y, Usui F, Morimoto J, et al. Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation.* 2011;123:594-604. doi:10.1161/CIRCULATIONAHA.110.982777

45. Bluthe RM, Pawlowski M, Suarez S, Parnet P, Pittman Q, Kelley KW, et al. Synergy between tumor necrosis factor alpha and interleukin-1 in the induction of sickness behavior in mice. *Psychoneuroendocrinology.* 1994;19(2):197-207.

46. Bluthe RM, Laye S, Michaud B, Combe C, Dantzer R, Parnet P. Role of interleukin-1beta and tumour necrosis factor-alpha in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice. *Eur. J. Neurosci.* Dec 2000;12(12):4447-4456.

47. Tu RH, Zeng ZY, Zhong GQ, Wu WF, Lu YJ, Bo ZD, et al. Effects of exercise training on depression in patients with heart failure: a systematic review and meta-analysis of randomized controlled trials. *Eur J Heart Fail.* Jul 2014;16(7):749-757. doi:10.1002/ejhf.101

48. Pozehl B, Duncan K, Hertzog M. The effects of exercise training on fatigue and dyspnea in heart failure. *European journal of cardiovascular nursing : journal of*

the Working Group on Cardiovascular Nursing of the European Society of Cardiology. Jun 2008;7(2):127-132. doi:10.1016/j.ejcnurse.2007.08.002

49. Kohut ML, McCann DA, Russell DW, Konopka DN, Cunnick JE, Franke WD, et al. Aerobic exercise, but not flexibility/resistance exercise, reduces serum IL-18, CRP, and IL-6 independent of beta-blockers, BMI, and psychosocial factors in older adults. *Brain. Behav. Immun.* May 2006;20(3):201-209. doi:10.1016/j.bbi.2005.12.002

50. Stensvold D, Slordahl SA, Wisloff U. Effect of exercise training on inflammation status among people with metabolic syndrome. *Metabolic syndrome and related disorders.* Aug 2012;10(4):267-272. doi:10.1089/met.2011.0140

51. Irwin MR, Olmstead R. Mitigating cellular inflammation in older adults: a randomized controlled trial of Tai Chi Chih. *Am. J. Geriatr. Psychiatry.* Sep 2012;20(9):764-772. doi:10.1097/JGP.0b013e3182330fd3

52. Christiansen T, Paulsen SK, Bruun JM, Pedersen SB, Richelsen B. Exercise training versus diet-induced weight-loss on metabolic risk factors and inflammatory markers in obese subjects: a 12-week randomized intervention study. *American journal of physiology. Endocrinology and metabolism.* Apr 2010;298(4):E824-831. doi:10.1152/ajpendo.00574.2009

53. Lin X, Zhang X, Guo J, Roberts CK, McKenzie S, Wu WC, et al. Effects of Exercise Training on Cardiorespiratory Fitness and Biomarkers of Cardiometabolic Health: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *J Am Heart Assoc.* Jul 2015;4(7). doi:10.1161/jaha.115.002014

54. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 2009;27:519-550.

55. Dinarello CA. A clinical perspective of IL-1b as the gatekeeper of inflammation. *Eur. J. Immunol.* 2011;41:1203-1217.

56. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood.* Apr 7 2011;117(14):3720-3732. doi:10.1182/blood-2010-07-273417

57. Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, et al. Evidence for altered interleukin (IL)-18 pathway in human heart failure. *The FASEB Journal.* 2004.

58. Toldo S, Mezzaroma E, O'Brien L, Marchetti C, Seropian IM, Voelkel NF, et al. Interleukin-18 mediates interleukin-1-induced cardiac dysfunction. *American journal of physiology. Heart and circulatory physiology.* Apr 1 2014;306(7):H1025-1031. doi:10.1152/ajpheart.00795.2013

59. Dinarello CA, Simon A, van der Meer J. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nature Reviews Drug Discovery.* 2012;11:633-652.

60. Novick D, Kim SH, Fantuzzi G, Reznikov LL, Dinarello CA, Rubinstein M. Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity.* Jan 1999;10(1):127-136.

61. Olee T, Hashimoto S, Quach J, Lotz M. IL-18 is produced by articular chondrocytes and induces proinflammatory and catabolic responses. *J. Immunol.* Jan 15 1999;162(2):1096-1100.

62. Feiereisen P, Vaillant M, Gilson G, Delagardelle C. Effects of different training modalities on circulating anabolic/catabolic markers in chronic heart failure. *Journal of Cardiopulmonary Rehabilitation and Prevention.* 2013;33:303-308.

63. Tsarouhas K, Tsitsimpikou C, Haliassos A, Georgoulias P, Koutsioras I, Kouretas D, et al. Study of insulin resistance, TNF-alpha, total antioxidant capacity and lipid profile in patients with chronic heart failure under exercise. *In Vivo.* Nov-Dec 2011;25(6):1031-1037.

64. Smart NA, Larsen AI, Le Maitre JP, Ferraz AS. Effect of exercise training on interleukin-6, tumour necrosis factor alpha and functional capacity in heart failure. *Cardiol Res Pract.* 2011;2011:532620. doi:10.4061/2011/532620

65. Byrkjeland R, Nilsson BB, Westheim AS, Arnesen H, Seljeflot I. Inflammatory markers as related to disease severity in patients with chronic heart failure: limited effects of exercise training. *Scand. J. Clin. Lab. Invest.* Nov 2011;71(7):598-605. doi:10.3109/00365513.2011.598943

66. Gatta L, Armani A, Iellamo F, Consoli C, Molinari F, Caminiti G, et al. Effects of a short-term exercise training on serum factors involved in ventricular remodelling in chronic heart failure patients. *Int. J. Cardiol.* Mar 22 2012;155(3):409-413. doi:10.1016/j.ijcard.2010.10.045

67. Prescott E, Hjardem-Hansen R, Dela F, Teisner AS, Nielsen H. Exercise training in older patients with systolic heart failure: adherence, exercise capacity, inflammation and glycemic control. *Scand. Cardiovasc. J.* Aug 2009;43(4):249-255. doi:10.1080/14017430802593427

68. Smart NA, Steele M. The effect of physical training on systemic proinflammatory cytokine expression in heart failure patients: a systematic review. *Congest Heart Fail.* May-Jun 2011;17(3):110-114. doi:10.1111/j.1751-7133.2011.00217.x

69. Bracey NA, Gershkovich B, Chun J, Vilaysane A, Meijndert HC, James R. Wright J, et al. Mitochondrial NLRP3 induces reactive oxygen species to promote Smad signaling and fibrosis independent from the inflammasome. *J. Biol. Chem.* 2014. doi:0.1074/jbc.M114.550624

70. Lindholm ME, Marabita F, Gomez-Cabrero D, Rundqvist H, Ekstrom TJ, Tegner J, et al. An integrative analysis reveals coordinated reprogramming of the epigenome and the transcriptome in human skeletal muscle after training. *Epigenetics.* Dec 2014;9(12):1557-1569. doi:10.4161/15592294.2014.982445

Table 4.1. Real-Time PCR Primers

Name	Forward	Reverse
GAPDH	5'-GCTTGAATCTAAATTATCAGTC-3'	5'-GAAGATTCAAATTGCATCTTAT-3'
ASC	5'-GCCGAGCTCACCGCTAACG-3'	5'-CATCCAGCAGCCACTCAACG-3'
IL-18	5’-CAAGGAAATCGGCCTCTATT-3’	5’-TCCTGGGACACTTCTCTGAA-3’
iNOS	5’-CAAGCCTACCCCTCCAGATG-3’	5’-CATCTCCCGTCAGTTGGTAGGT-3’

GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

ASC = Apoptosis-associated speck-like protein containing a caspase recruitment domain

IL-18 = Interleukin-18

iNOS = Inducible nitric oxide synthase

Table 4.2. Baseline Characteristics for the Total Sample and by Group

Measure	Total Sample (N=54)	Control Group (n=16)	Exercise Group (n=38)	Differences (p-value)[1]
Age (years) mean (SD)	59.46 (9.7)	58.19 (12.8)	60.0 (8.7)	.54
Gender, n (%) Male Female	26 (48%) 28 (52%)	5 (31%) 11 (69%)	21 (55%) 17 (45%)	.11
Race, n (%) AA/Black Non-black	32 (59%) 22 (41%)	11 (69%) 5 (31%)	21 (55%) 17 (45%)	.36
LVEF, %, mean (SD)	33.67 (14.9)	35.53 (12.7)	32.85 (15.9)	.55
BMI (kg/m2), mean (SD)	31.37 (6.8)	31.03 (6.1)	31.51 (7.1)	.81
6MWT (m), mean (SD)	348.90 (78.2)	330.97 (84.8)	356.45 (12.2)	.28
Peak $\dot{V}O_2$, mean (SD)	16.68 (4.7)	15.73 (4.3)	17.02 (4.8)	.40
CCI, mean (SD)	3.84 (1.8)	3.47 (2.1)	4.0 (1.7)	.35
Education, n (%) ≤ HS ≥ College	26 (48%) 28 (52%)	8 (50%) 8 (50%)	18 (47%) 20 (53%)	.90
β-Blocker, n (%) No Yes	10 (13%) 44 (87%)	4 (25%) 12 (75%)	6 (16%) 32 (84%)	.43
Type of HF HFpEF HFrEF	19 (35%) 35 (65%)	6 (38%) 13 (62%)	10 (26%) 23 (74%)	.92

[1]p-value of group differences from Student's t-test for continuous variables and chi-square for categorical variables.

AA – African American

LVEF – Left ventricular ejection fraction

BMI – Body mass index

6MWT – Six-minute walk test

CCI – Charlson Comorbidity Index

HS – High school

HFpEF – Heart failure with preserved ejection fraction

HFrEF – Heart failure with reduced ejection fraction

Table 4.3. Mean Percent ASC Methylation, IL-1β, IL-18, and TNFα by Group[a]

	% ASC methylation Mean (SD)	IL-1β (pg/mL) Mean (SD)	IL-18 (pg/mL) Mean (SD)	TNFα (pg/mL) Mean (SD)
Baseline				
Control (n=16)	5.75 (0.6)	1.88 (1.0)	162.67 (62.1)	1.37 (0.5)
Exercise (n=38)	5.88 (0.9)	1.65 (0.8)	149.90 (33.8)	1.61 (0.5)
3 Months				
Control (n=13)	5.80 (0.4)	2.09 (1.3)	162.86 (66.8)	1.40 (0.4)
Exercise (n=24)	6.10 (0.5)*†	1.43 (0.5)*†	138.18 (56.0)	1.43 (0.5)
6 Months				
Control (n=11)	5.82 (0.4)	2.13 (1.4)	160.58 (34.7)	1.48 (0.5)
Exercise (n=18)	6.07 (0.4)*†	1.49 (0.5)*‡§	130.15 (52.9)	1.25 (0.5)

[a]PROC MIXED with least squares mean analysis adjusting with Bonferroni was used to compare within GROUP and between GROUP changes across TIME.

*Group differences significant at $p<.05$.

†Difference from baseline is significant at $p<.01$.

‡ Difference from baseline significant at $p<.05$.

§Difference from 3 months significant at $p<.05$.

Table 4.4. Multilevel Modeling for Entire Sample (N=54)

	Variable	Coefficient	SE	p-value
	ASC Methylation			
Fixed Effects				
	Intercept	5.64	0.13	<.001
	LVEF[a]	0.2	0.01	.05
	Peak $\dot{V}O_2$[a] x Gender[b]	0.02	0.01	.01
Rate of Change				
	Intercept	-0.10	0.01	.06
	Group[c]	0.3	0.01	.006
	Interleukin-1β			
Fixed Effects				
	Intercept	1.96	0.21	<.001
	LVEF[a]	-0.1	0.004	.05
	Peak $\dot{V}O_2$[a] x Gender[b]	-.04	0.01	.006
Rate of Change				
	Intercept	-0.11	0.04	0.02
	Group[c]	-0.11	0.04	.02
	Completed Intervention[d]	-0.63	0.02	.006

[a]LVEF (%) and peak $\dot{V}O_2$ (ml/kg/min) were mean-centered for analysis

[b]0=Female, 1=Male

[c]0=Control Group, 1=Exercise Group

[d]0=Did not complete 3-month intervention, 1=Exercised for 3 months

LVEF – Left ventricular ejection fraction

$\dot{V}O2$ – Oxygen uptake

Table 5. Multilevel Modeling for Exercise Group Only (n=38)

	Variable	Coefficient	SE	p-value
	ASC Methylation			
Fixed Effects				
	Intercept	3.99	0.56	<.001
	LVEF[a]	0.13	0.09	.049
	Peak $\dot{V}O_2$[a] x Gender[b]	0.11	0.03	.002
Rate of Change				
	Intercept	-0.02	.001	.051
	Gender[b]	0.04	0.01	.008
	Race[e]	-0.06	0.01	.004
	College[f]	0.04	0.01	.021
	Interleukin-1ß			
Fixed Effects				
	Intercept	1.96	0.21	<.001
	LVEF[a]	-.01	0.004	.050
	Peak $\dot{V}O_2$[a] x Gender[b]	-.04	0.01	.006
Rate of Change				
	Intercept	-0.11	0.04	.016
	Completed Intervention[d]	-0.64	0.02	.010

[a]LVEF (%) and peak $\dot{V}O_2$ (ml/kg/min) were mean-centered for analysis

[b]0=Female, 1=Male

[c]0=Control Group, 1=Exercise Group

[d]0=Did not complete 3-month intervention, 1=Exercised for 3 months

[e]0=African American/Black 1=Caucasian

[f]0=Did not attend college 1=Attended college

LVEF – Left ventricular ejection fraction

$\dot{V}O2$ – Oxygen uptake

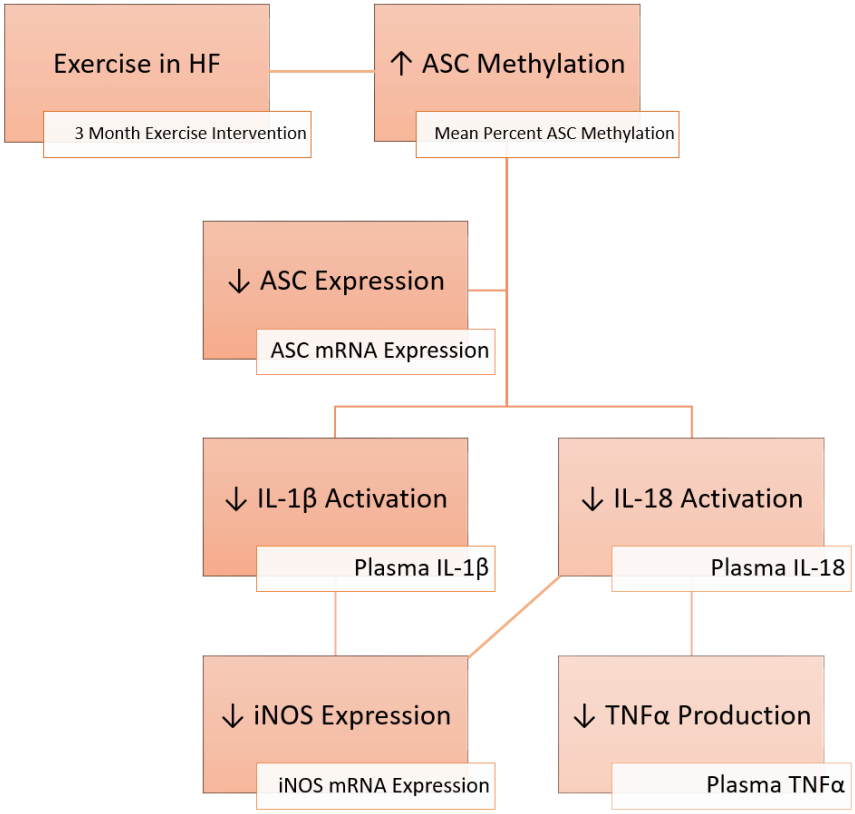

Figure 4.1. Proposed relationships related to inflammatory changes after exercise in persons with heart failure. We proposed that a 3-month exercise intervention in persons with heart failure would be related to increased apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) methylation. Further, we propose that this increase in ASC methylation would be related to decreased ASC mRNA, decreased IL-1β, and decreased IL-18. IL-18 is thought to increase production of TNFα, and both IL-18 and IL-1β are associated with increased expression of inducible nitric oxide synthase (iNOS). Therefore, we proposed that after an exercise intervention

decreased IL-18 would be related to decreased TNFα and decreased iNOS expression and decreased IL-1β would be related to decreased iNOS expression.

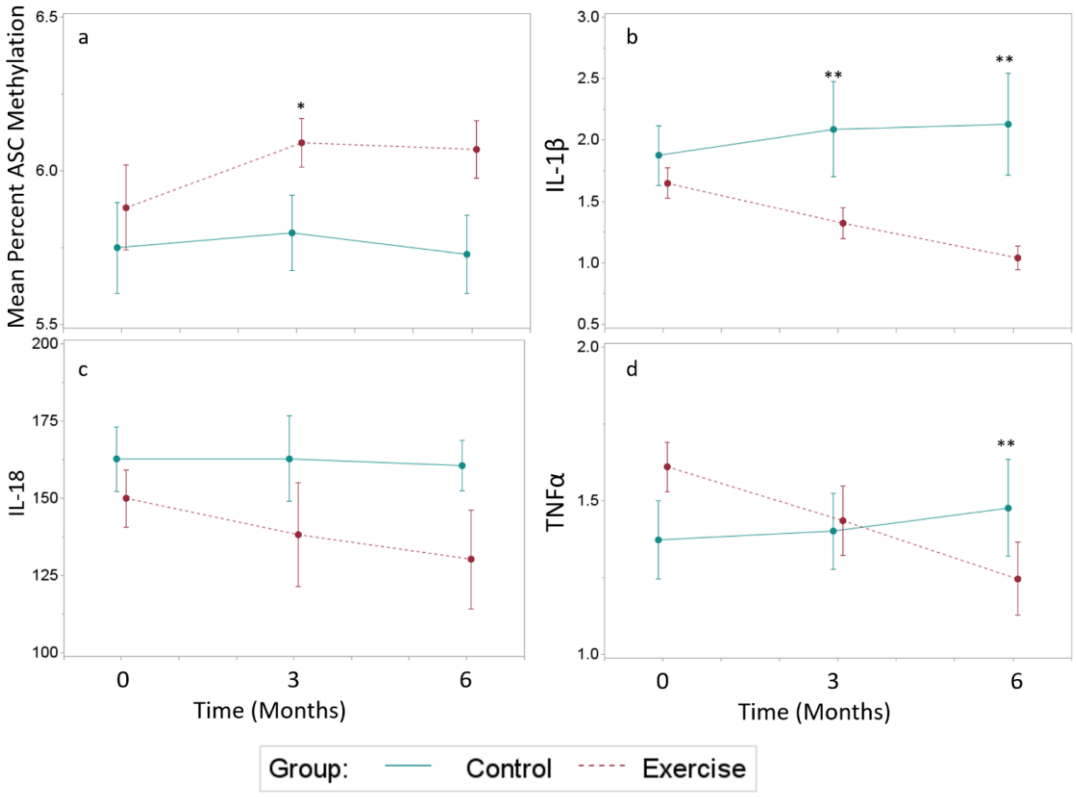

Figure 4.2. Changes in Mean Percent ASC Methylation, IL-1β, IL-18, and TNFα over Time by Group.

a. Group differences in change scores for ASC methylation were found from baseline to 3 months (control 0.04% vs. exercise -0.04%, t=-2.71, p=.011). These changes were sustained at 6 months for the exercise group. b. Group differences in change scores for IL-1β were found from baseline to 3 months (control 0.18 pg/mL vs exercise -0.33 pg/mL, t=3.73, p=.001).These changes were sustained at 6 months for the exercise group. c. No group differences in change scores over time were found for IL-18. d. Group differences in change scores for TNFα were found from baseline to 6 months (control 0.23 pg/mL vs exercise -0.22 pg/mL, t=2.79, p=.01).

*Significant group differences in change from baseline at p<.05

**Significant group differences in change from baseline at p<.01

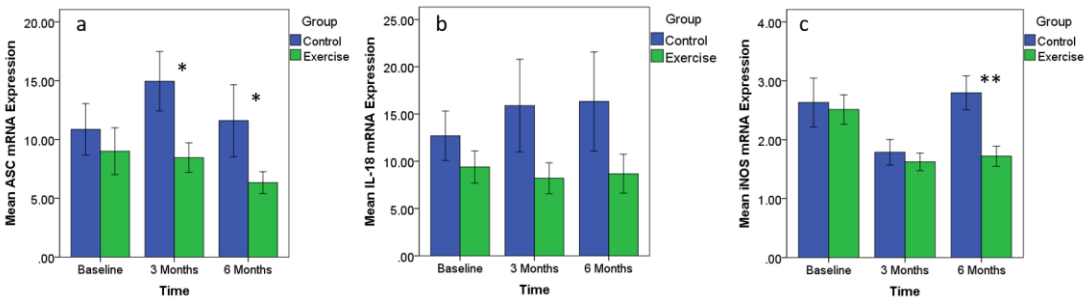

Figure 4.3. Mean ASC, IL-18, and iNOS mRNA Expression by Group over Time[1]

PROC MIXED with least squares mean analysis adjusting with Bonferroni was used to compare within GROUP and between GROUP changes across TIME. To normalize gene expression relative to a non-HF reference sample, mRNA quantification results were calculated using the $2^{-\Delta\Delta CT}$ method.[35] Values are presented as mean ± SD. a. There were significant group differences in relative ASC mRNA expression at 3 months (t=2.27, p=.027) and 6 months (t=2.24, p=.029). There were within-group decreases in relative ASC mRNA expression from baseline to 3 months (t=3.01, p=.004) and from 3 months to 6 months (t=4.82, p=.001) for the exercise group. b. There were no significant inter- or intra-individual changes in relative IL-18 mRNA expression over time. c. There were significant group differences in relative iNOS mRNA expression at 6 months (t=3.51, p=.009). There were within-group decreases in relative iNOS mRNA expression from baseline to 3 months (t=3.32, p=.002), and the decrease was sustained at 6 months (t=2.65, p=.01) in the exercise group and from baseline to 3 months in the control group (t=2.33, p=.02). Relative iNOS mRNA expression returned to baseline levels in the control group by 6 months.

[1]mRNA gene expression was normalized relative to a reference sample (set at a value of 1).

[*]Significant group differences at p=.03

[**]Significant group differences at p=.01

Chapter V

Summary and Conclusions

The purpose of this study was to examine the effects of aerobic exercise on changes in ASC (apoptosis-associated spec-like protein containing a caspase recruitment domain) methylation and to determine whether this initiated a downstream change in inflammatory cytokines (interleukin-1β [IL-1β], interleukin-18 [IL-18]) known to worsen outcomes in persons with heart failure (HF). The specific aims of the study were to examine the effects of an aerobic exercise intervention on ASC methylation in persons with heart failure, to examine the relationship of ASC methylation and inflammatory cytokines in persons with HF, and to examine relationships between percent ASC methylation, plasma IL-1β, and plasma IL-18 with aerobic capacity in persons with HF.

HF is associated with a chronic low-grade inflammation leading to adverse cardiac remodeling and disease progression.[1] This chronic inflammation is characterized by the formation and activation of an intracellular protein complex, the inflammasome, which in turn activates inflammatory cytokines that promote cardiac hypertrophy and myocardial apoptosis.[1-3] Increased inflammatory cytokines are associated with increased HF progression, severity, and death.[4-7]

Because HF remains a leading cause of morbidity and mortality in the United States,[8,9] identification of novel therapeutic targets that may slow disease progression are urgently needed. Modification of the inflammasome has been proposed as a lower-risk alternative to current pharmacological trials targeting reduction of circulating cytokines, but only pharmacological have been reported to date.[10,11] Therefore, this study proposed

a non-pharmacological, modifiable molecular pathway, ASC methylation, as an aerobic exercise intervention target for persons with HF.

In the preliminary study for this project, stored samples from 155 HF outpatients (mean age 56.9 ± 12.0 years, 36% female, 47% African American, and mean ejection fraction 29.9 ± 14.9%) were analyzed for percent methylation of seven CpG sites in the intron region preceding exon-1 of the ASC gene. ASC methylation was inversely related to ASC mRNA and protein, and ASC gene expression was positively related to IL-1β (Figure 5.1). ASC methylation had a positive linear relationship with ejection fraction ($r=.85$, $p<.001$), quality of life ($r=.83$, $p<.001$), and six-minute walk test ($r=.59$, $p=.023$), and a negative linear relationship with depression ($r=-.81$, $p<.001$) and anxiety ($r=-.75$, $p<.001$). Higher ASC methylation was associated with a lower risk for clinical events (HR 0.16, $p=.025$) while higher protein (HR=1.78, $p=.045$) and mRNA expression (HR=1.18, $p=.05$) were associated with a greater risk.[12]

The preliminary study was the first study to examine ASC methylation and outcomes in persons with HF. The preliminary data from that study demonstrated that increased ASC methylation is a likely contributor to the pathophysiology of HF and outcomes, and provided a foundation for the aims of this dissertation study.

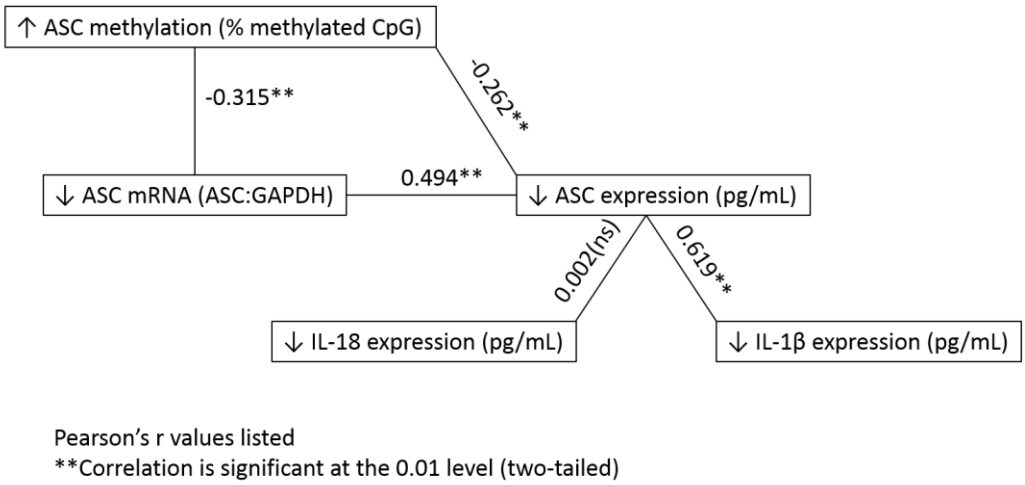

Figure 5.1. Association Between ASC Methylation, ASC Expression, and Cytokine Expression Increased percent methylation of 7 CpG sites immediately preceding exon 1 of ASC is related to decreased ASC mRNA and protein expression. Decreased ASC expression is significantly related to decreased IL-1β expression. No significant relationship was found between ASC expression and IL-18 expression.

ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

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The three manuscripts (Chapters 2-4) included in this dissertation outline the evidence for inflammasome involvement in HF pathophysiology (Chapter 2), describe the relationship between aerobic capacity and ASC methylation in HF (Chapter 3), and demonstrate that an aerobic exercise intervention increases ASC methylation while

decreasing inflammasome-activated cytokines in persons with HF (Chapter 4). This research is the first to examine the effects of aerobic exercise on changes in DNA methylation in persons with HF and provides the first evidence that exercise may be able to reverse epigenetic-induced changes in gene expression of inflammatory proteins and other markers associated with HF pathophysiology, thereby improving outcomes.

The first aim of this study examined the effects of exercise on ASC methylation in persons with heart failure (N=54) and is addressed in Chapter 4. The hypothesis of this aim was that persons with HF (mean age 59.5 ± 9.7 years, 52% female, 59% African American, and mean ejection fraction 33.67 ± 6.8%), who participated in a 3-month exercise intervention, would have increased ASC methylation at the completion of the intervention as compared to persons with HF in an attention-control group. The exercise intervention consisted of a progressive, moderate-intensity walking program in which participants were instructed to walk for 30 minutes three times per week for the first two weeks followed by 45 minutes three times per week for the remaining ten weeks. Each participant was provided with an individualized exercise prescription based on maximum heart rate obtained during a symptom-limited, modified Balke treadmill test at baseline. The attention control group received education and flexibility and stretching exercises. A 3-month exercise intervention in persons with HF was associated with increased ASC methylation at 3 months with medium to large effect sizes, supporting this hypothesis. The increase in ASC methylation after exercise was associated with a decrease in ASC mRNA expression, suggesting that exercise may decrease inflammation in HF via epigenetic control of inflammasome formation due to decreased bioavailability of ASC.

This aim also examined ASC methylation at 6 months to determine if the intervention effects on ASC methylation were sustained at 6 months, which was 3 months after completing the exercise intervention. Indeed, the changes in ASC methylation remained higher than baseline at 6 months among participants in the exercise group and was significantly higher than ASC methylation in the control group. ASC methylation at 6 months was not significantly higher than ASC methylation at 3 months. During the time from 3 months to 6 months, participants were in a 'maintenance' period, in which they were no longer actively involved in the intervention. We did not capture the level of exercise during the 3-month maintenance phase, so we were unable to identify if changes sustained at 3 months were due to continued exercise or if there were short-term lasting epigenetic effects after the exercise intervention. There may also be an upper limit to the level of ASC methylation, even with sustained physical activity, limiting the changes in ASC methylation with continued exercise.

The second aim of this study examined the relationships between ASC methylation and inflammatory cytokines in persons with HF and is partially addressed in Chapter 4. This aim hypothesized that increased ASC methylation would be related to decreased plasma IL-1β, IL-18, and TNFα and with decreased iNOS mRNA expression in persons with HF. Increased ASC methylation was related to decreased IL-1β at baseline, 3 months, and 6 months but not to IL-18 or TNFα. ASC methylation was also negatively related to iNOS mRNA expression over time, and this relationship was mediated by IL-1β. Changes in IL-1β over time were related to changes in IL-18 and TNFα from baseline to 3 months and from baseline to 6 months. This hypothesis was

partially supported, in that increased ASC methylation was associated with decreased IL-1β and iNOS, but not with IL-18 and TNFα. It is possible that the effects of exercise led to decreased formation of danger-associated molecular patterns (DAMPs), the impetus for inflammasome formation and activation, independent of changes in ASC methylation. However, the associations between ASC methylation, ASC mRNA expression, and IL-1β before and after the exercise intervention suggest the changes in ASC methylation likely decrease inflammasome formation and function, at least, to some extent. These data suggest that the reduction in inflammatory cytokines after an exercise intervention occur via decreased activation of IL-1β and that this reduction in IL-1β is related to epigenetic control of ASC production.

The second aim also contained two research questions not addressed in the previous chapters: 1. Does increased ASC methylation, in response to the exercise intervention, triggers IL-18 activation, as evidenced by changes in the ratio of proform IL-18 mRNA to plasma IL-18, and 2. Does increased ASC methylation have a *mediating* effect on changes in circulating IL-1β and IL-18 in persons with HF? The results of these research questions are discussed here.

Unlike IL-1β, IL-18 is constitutively expressed in peripheral blood cells.[13] To examine if changes in inflammasome activity were related to changes in IL-18 activation, the ratio of IL-18 mRNA to circulating IL-18 was analyzed. No associations were found between changes over time in the IL-18 mRNA:protein ratio and changes in ASC methylation or ASC mRNA. However, significant positive associations were found between changes in the IL-18 mRNA:protein ratio and changes in IL-1β from baseline to

6 months (r=.51, p=.006) and from 3 months to 6 months (r=.55, p=.004). Because there were no significant changes in IL-18 mRNA over time, this data is most likely reflective of the relationship between changes in protein levels over time. The ratio is calculated by dividing mRNA expression by protein levels. If there are no significant changes over time in mRNA expression levels (numerator), any changes in the ratio values are thus due to changes in the protein levels (denominator).

The second research question looked at the mediating effect of ASC methylation on changes in circulating IL-1β and IL-18 in persons with HF. This research question was purely exploratory and was underpowered for analysis. Using the PROCESS macro, a mediation model was fit to examine the effect of the increased ASC methylation at 3 months in the relationship of IL-1beta at baseline and 3 months (Figure 5.2). Increased ASC methylation at 3 months *mediates* the relationship between IL-1β at baseline and IL-1β at 3 months, controlling for gender, race, left ventricular ejection fraction, peak $\dot{V}O_2$, and education, evidenced by the significant indirect effect and the decrease in strength of the relationship in the mediation model as compared to the direct effect. No mediation effect was found for IL-18.

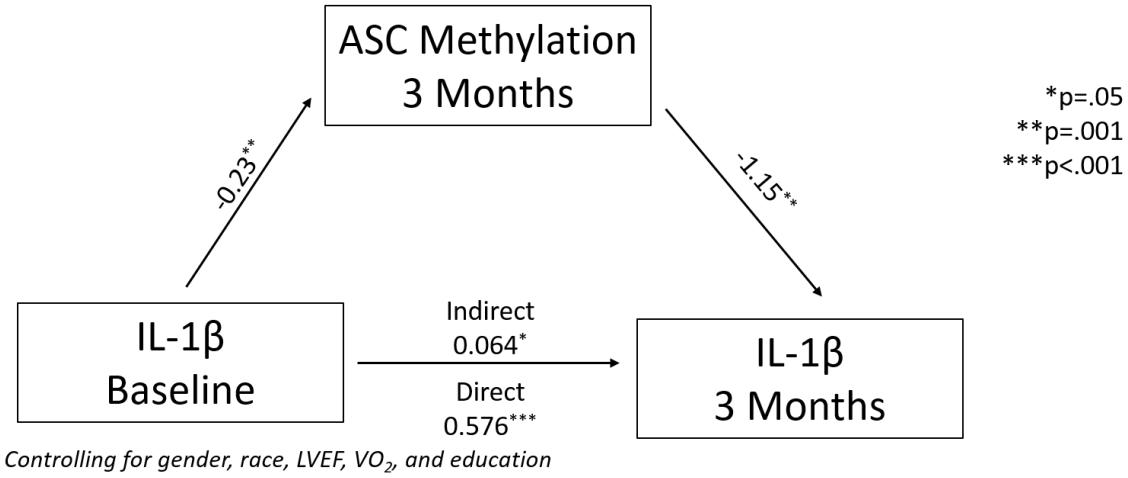

Figure 5.2. Increased ASC Methylation Has a Mediating Effect on Interleukin-1β

ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain

LVEF: left ventricular ejection fraction

$\dot{V}O_2$: peak oxygen consumption

ASC methylation was negatively associated with IL-1β at baseline, 3 months, and 6 months. Similarly, ASC mRNA expression was positively associated with IL-1β at baseline, 3 months, and 6 months. Although this study could not demonstrate that the changes in IL-1β were directly related to changes in ASC methylation and expression in response to an exercise intervention, the strong associations over time are suggestive of an interrelated pathway. ASC-deficient mice are unable to activate IL-1β or IL-18 upon LPS stimulation, demonstrating that ASC is necessary for caspase-1 dependent IL-1β and IL-18 activation.[14-16] ASC availability is likely a limiting factor in inflammasome formation, and thus IL-1β and IL-18 activation, leading to the conclusion that decreased

ASC gene expression via increased DNA methylation is a direct cause of decreased IL-1β activation.

IL-18 fails to induce systemic inflammatory events, and function of IL-18 may be more important at tissue-specific level.[17] IL-18 is produced by cardiac cells in response to ischemic-reperfusion injury and stimulates cardiac extracellular matrix remodeling.[6] In circulation, IL-18 can induce Th1 polarization. Additionally, IL-18 is thought to be produced by vascular cells in response to stress or hypoxia.[18] The pleiotropic activities of IL-18 may account for the mixed results in this study.

IL-18 is regulated by an endogenous inhibitor, IL-18 binding protein (IL-18BP), which inactivates IL-18 when bound.[19] This IL-18/IL-18BP pathway is altered in the failing myocardium and may account for the lack of association with IL-1β in this study over time. This study did not examine IL-18 production but rather circulating IL-18. Therefore, the data presented in this study may not have captured all activated IL-18, only that which had been released from cells. In addition, this study did not measure IL-18BP, so the dynamic nature of IL-18 activity was not analyzed.

The third aim of this study was exploratory and is addressed in Chapter 3. The purpose of this aim was to examine the relationships between percent ASC methylation, plasma IL-1β, and plasma IL-18 with aerobic capacity, as measured by peak $\dot{V}O_2$, in persons with HF. Mean percent ASC methylation and plasma IL-1β levels were associated with clinically meaningful changes over time in peak $\dot{V}O_2$ in persons with HF. The positive association of ASC methylation and peak $\dot{V}O_2$ in this study adds a putative

mechanism of epigenetic control of inflammatory gene expression contributing to decreased aerobic capacity in HF.

IL-1β has been shown to affect β-adrenergic receptor responsiveness *in vitro*, and may be a key determinant of exercise capacity in HF.[20] Studies in both HFrEF and HFpEF demonstrated that a two-week treatment with an IL-1β blockade improved aerobic capacity without any increase in physical activity.[20,21] Combined with the data from this study, level of ASC methylation may directly affect aerobic capacity in HF. Further work examining this effect is needed.

A previous study by Nakajima *et al*.[22] found no relationship between ASC methylation and $\dot{V}O_{2max}$ in their study of healthy adults. This difference in findings in the current study may be related to the pathophysiological and structural changes that limit functional capacity in HF. In HF, aerobic capacity is measured as peak $\dot{V}O_2$, as opposed to the $\dot{V}O_{2max}$ measurement in the healthy population. The measurement of $\dot{V}O_{2max}$ requires an individual to reach a plateau of maximum volume of oxygen used; persons with HF are rarely able to reach and sustain this plateau. Peak $\dot{V}O_2$ is reduced primarily by impaired cardiac output in both heart failure with reduced ejection fraction (HFrEF) and preserved ejection fraction (HFpEF).[23] Other factors such as endothelial dysfunction leading to vasoconstriction and decreased capacity for aerobic metabolism contribute to the reduction in maximum oxygen uptake in HF.[23] These physiological processes are amplified by a positive feedback loop of inflammation and may explain, at least in part, the association between peak $\dot{V}O_2$ and the inflammasome-related measures. Further investigation comparing these relationships to age and gender matched healthy controls is needed.

Increased cytokines in HF may contribute to the pathophysiology and disease progression by altering cardiac structure and function. In addition, inflammatory cytokines may also contribute to peripheral alterations in vascular and skeletal muscle function. The cardiovascular, respiratory, and skeletal muscle systems all influence aerobic capacity,[23] and the individual contributions of pathophysiological changes in each system to impaired aerobic capacity are difficult to discern. This study demonstrated a relationship between IL-1β and peak $\dot{V}O_2$ in a systemic source (plasma), but further investigation into the relationships and mechanisms of inflammatory cytokines in cardiovascular and skeletal muscle tissue is warranted.

Low aerobic capacity is a powerful predictor of premature morbidity and higher mortality in HF,[24] and adding IL-1β levels may provide better prediction of adverse outcomes in HF. The preliminary work for this study demonstrated that ASC methylation, protein, and mRNA expression are predictors of clinical events in HF.[12] These data further implicate inflammasome pathway in the pathophysiological processes of HF. Modification of inflammation via epigenetic modulation may be a novel target for intervention in HF.

An interesting finding from this study was that the relationship between peak $\dot{V}O_2$ and IL-18 was found only in the HFrEF (LVEF $\leq$ 40%) subset. The pathophysiology of HFrEF is distinct from that of HFpEF, and this finding may reflect the role of IL-18 in the pathophysiology of HFrEF. Studies examining IL-18 in HF have primarily focused on post-ischemic ventricular changes or dilated cardiomyopathy related to systolic dysfunction. Alterations in the IL-18/IL-18BP pathway are thought to occur early in the pathophysiological processes of HF, and are reflective of an HFrEF phenotype. HFpEF

is caused by multiple impairments, related to myriad factors such as diastolic reserve function, heart rate reserve, rhythm abnormalities, atrial dysfunction, and stiffening of the ventricles and vasculature.[25-28] The cellular changes in the myocardium alter structure and function differently in HFpEF and HFrEF, leading to different phenotypes.[29] Thus, while inflammatory processes are upregulated in both HFpEF and HFrEF, it is likely that specific inflammatory pathways differ. Overall, studies comparing biomarkers of HFpEF and HFrEF are limited. Further examination of changes in gene expression and signaling pathways may provide insights into the pathophysiology and new targets for treatment in this poorly understood syndrome.

Many studies of DNA methylation changes with exercise just examine genome-wide methylation and do not examine pathways or changes in gene expression.[30-34] Importantly, these studies do not address the effects of changes in DNA methylation or what mechanisms are affected by these exercise-induced epigenetic alterations. DNA methylation can increase or decrease with different outcomes, depending on where in the gene region the methylation is located. As in this study, DNA methylation of CpG islands in the promoter region generally silences gene expression.[11] In contrast, CpG methylation in exon regions often upregulates gene expression.[30] Therefore, studies examining specific gene regions that are accompanied by gene expression can shed more light on how exercise leads to epigenetic control of physiological or pathophysiological mechanisms.

A meta-analysis of changes in DNA methylation associated with exercise found that effect size of DNA methylation change across 16 different publications and 1580

people was large, with a Cohen's d of 1.20.[30] Further, they found that the effect size of DNA methylation change with exercise was greater for participants over 40 years of age as compared to participants under 40 years, particularly with regard to studies measuring increases in DNA methylation.[30] Genome-wide DNA methylation has been shown to decrease with age,[35-38] and thus, the epigenetic protective effects of exercise are likely more apparent in the face of the age-related changes in DNA methylation of older adults as compared to the younger population. In this study, we only included participants who were at least 40 years of age, and our hypothesis was to find an increase in DNA methylation of ASC after the exercise intervention. Thus, combined with the previous evidence demonstrating higher ASC methylation among older adults (≥ 40 years of age) who exercised as compared to a control group,[22] the medium to large effect sizes related to changes in DNA methylation in this study were similar to previous studies.

This study found systemic changes in DNA methylation, gene expression, and circulating cytokines after an aerobic exercise intervention in persons with HF. However, more meaningful changes after exercise may occur at the tissue level, especially in skeletal muscle, and these changes may significantly impact overall aerobic and functional capacity in HF. HF is associated with muscle wasting in the absence of weight loss.[39] This loss of skeletal muscle contributes to increased fatigability, decreased endurance, and decreased aerobic capacity in HF.[40] Circulating cytokines produced by the failing heart likely affects skeletal muscle function,[40] but what mediates crosstalk between cardiac tissue and skeletal muscle is currently unknown. This study measured plasma cytokines IL-1β, IL-18, and TNFα, but the tissue of origin for these circulating cytokines could be from multiple tissues. The aerobic exercise intervention may have

affected the release of these cytokines differently in the various tissues or cell types, such as resident cardiac macrophages, peripheral mononuclear cells, skeletal muscle, or cardiac fibroblasts. Exercise has been shown to reduce TNFα, IL-1β, and iNOS in skeletal muscle of persons with HF,[41,42] but the effects of exercise on expression of these cytokines in the failing heart is largely unknown. Further research into the dynamics of inflammasome activation and cardiac cross-talk with skeletal muscle after an exercise intervention in persons with HF is warranted.

Limitations and Strengths

This study was the first study to examine epigenetic changes with exercise in persons with HF and used a short-term exercise program (12 weeks). Previous studies have demonstrated the beneficial effects of a short-term exercise intervention in persons with HF.[43-46] Longer interventions may provide more insight into the dynamics of changes in inflammatory proteins over time and examine the limits and rate of change of DNA methylation with exercise. The diverse sample in this study enhances the generalizability to a larger HF population.

We did not capture the level of exercise during the 3-month maintenance phase, so we were unable to identify if changes are sustained at 3 months were due to continued exercise or if there are short term lasting epigenetic and anti-inflammatory effects. Exercise adherence was self-report, and some participants did not provide a self-report exercise log. Thus, we may have over or underestimated actual adherence. Real-time activity trackers may provide more objective data to better identify the relationships between exercise intensity and endurance with changes in DNA methylation and cytokine

expression. The results of this study are specific to the 3-month walking exercise program and may not be generalizable to all types and durations of exercise.

This study measured DNA methylation and mRNA expression in peripheral blood mononuclear cells (PBMCs) and circulating cytokines in plasma. We did not measure changes in other tissues. There may be meaningful tissue-specific epigenetic changes with exercise resulting in altered inflammatory profile in the myocardium and skeletal muscle, and these changes may more tightly align with changes in cytokine expression. We used PBMCs collected from whole blood and did not control for leukocyte differential. Intra-individual and inter-individual differences over time may be related to different leukocyte composition that we were unable to capture.

The aim examining the relationships between $\dot{V}O_2$ and ASC methylation was exploratory and was cross-sectional with a relatively small sample size of 54 participants. A larger study following changes in $\dot{V}O_2$ and ASC methylation over time may shed more light on the dynamic relationship between these measures. While peripheral blood mononuclear cells (PBMCs) and serum cytokine levels are indicative of systemic inflammation, including cardiac and/or skeletal muscle biopsy samples would provide more insight into localized inflammatory changes affecting aerobic capacity. Further, healthy controls were not included in the study for comparison.

Only one study has been published to date that examined the relationship between ASC methylation and exercise;[22] that study did not examine changes in ASC methylation before and after an exercise intervention, but rather compared DNA methylation of ASC after an exercise intervention to a control group that had not exercised. This study was the first to examine changes in ASC methylation before and after an exercise intervention

and was the first study to examine changes in DNA methylation in response to exercise in persons with HF and to examine the effects in comparison with a control group.

Although the sample size of 60 provided adequate power for the questions set forth in this proposal, attrition of 14% throughout the study resulted in a lower sample size over time. Multilevel linear modeling was used to analyze group effects over time. This analysis can account for attrition, allowing for all time points collected to be included in the model. In addition, this study was a preliminary study to explore the effects of exercise on the inflammatory pathway, and the results serve to establish relationships, effect sizes, and will inform future work.

Implications for Future Research and Practice

HF is the leading cause of morbidity and mortality in the world, with a prevalence that is projected to increase over time.[8] The economic burden of outpatient and inpatient HF care is increasing in step with the increasing prevalence,[9] heightening the need for effective HF therapies. The role of exercise in HF may prove to be a more important part of HF management than currently realized. Examining molecular targets implicated in the pathological disease processes of worsening HF are vital to our understanding of exercise therapy in HF. Furthermore, uncovering novel molecular pathways in HF that can be modified by aerobic exercise may provide us with therapeutic targets for future HF interventions.

Over the past two decades, advances in pharmacological and device therapies for HF have significantly improved the prognosis for persons with HF. However, despite these advances in HF therapies, the 5-year mortality remains at 50%.[47] HF is the leading

cause of hospitalization among individuals over age 65, leading to costs of care exceeding 31 billion dollars annually.[9] Once hospitalized, persons with HF have a 30% risk of dying within 1 year, regardless of LVEF.[48] Therefore, attenuating HF disease progression remains an important goal. Identification of novel pathways and effectively intervening on potential therapeutic targets may slow HF disease progression.

Exercise is a low-priced, easily accessible HF treatment that has significant positive social and economic implications. Exercise has demonstrated both physical and psychological benefits in HF.[43,49,50] However, the plasticity of exercise-induced changes and its longer-term impact on inflammation in the context of HF outcomes could be largely dependent on epigenetic modification. Further research examining the effects of exercise in persons with HF on epigenetic control of key pathophysiologic pathways can serve to identify long-term benefits of exercise and relate these epigenetic changes to physical, psychological, economic, and quality of life outcomes in persons with HF.

This study demonstrated that a short-term (12 week) walking exercise intervention has significant epigenetic changes in HF that are related to decreased inflammation. The exercise prescription in this study was adequate for these changes, and may be used to inform exercise recommendations in practice. This study tested one exercise protocol that was prescribed for 90 minutes per week at 60% intensity for two weeks, followed by 135 minutes per week at 60% intensity for two weeks, then progressing to 135 minutes per week at 70% intensity for the remaining five weeks. Further studies testing other exercise strategies may provide further insight into the dose-response relationship between aerobic exercise and epigenetic control of inflammation of persons with HF.

Summary

The purpose of this study was to examine the effects of exercise on ASC methylation and IL-1 inflammatory cytokines in persons with HF. The main outcomes of this study demonstrated that a 3-month exercise intervention increased ASC methylation and decreased inflammatory markers in persons with HF. In addition, this study demonstrated that ASC methylation and IL-1β are associated with aerobic capacity in HF, providing further insight into the pathophysiological mechanisms in HF.

These findings further implicate the inflammasome as a key mediator of inflammation in HF and suggest that exercise may modulate inflammasome formation and/or activation. The moderate to large effect sizes found for changes in ASC methylation after a 3-month exercise intervention were promising results and require replication in a larger sample size, a longer intervention, and with more time points. The strong relationship between ASC methylation/expression and IL-1β over time suggests that the epigenetic changes that occur with exercise also reduce IL-1β in HF. However, more meaningful changes may occur in skeletal muscle, and this should be examined in future studies. Epigenetic regulation of ASC can be a biological mechanism by which exercise can promote better outcomes in HF. Further research examining mechanisms of change can lead to improved understanding of physiological adaptations and more precise prediction of adverse outcomes in persons with HF.

References

1. Abbate A. The heart on fire: Inflammasome and cardiomyopathy. *Exp. Physiol.* 2013;98(2):385. doi:10.1113/expphysiol.2012.069021
2. Bracey NA, Beck PL, Muruve DA, Hirota SA, Guo J, Jabagi H, et al. The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1β. *Exp. Physiol.* 2013;98(2):462-472. doi:10.1113/expphysiol.2012.068338
3. Paulus WJ, Tschöpe C. A novel paradigm for heart failure with preserved ejection fraction. *J. Am. Coll. Cardiol.* 2013;62(4):263-271.
4. Yamaoka-Tojo M, Tojo T, Inomata T, Machida Y, Osada K, Izumi T. Circulating levels of interleukin 18 reflect etiologies of heart failure: Th1/TH2 cytokine imbalance exaggerates the pathophysiology of advanced heart failure. *J. Card. Fail.* 2002;8(1):21-27.
5. Eslick GD, Thampan BV, Nalos M, McLean AS, Sluyter R. Circulating interleukin-18 concentrations and a loss-of-function P2x7 polymorphism in heart failure. *Int. J. Cardiol.* 2009;137(1):81-83. doi:10.1016/j.ijcard.2008.05.017
6. Hedayat M, Mahmoudi MJ, Rose NR, Rezaei N. Proinflammatory cytokines in heart failure: double-edged swords. *Heart Failure Reviews.* 2010;15:543-562.
7. Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, et al. Evidence for altered interleukin (IL)-18 pathway in human heart failure. *The FASEB Journal.* 2004.

8. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics - 2013 update: A report from the American Heart Association. *Circulation.* 2013;127:e6-e245.

9. Heidenreich PA, Trogdon JG, Khavjou OA, Butler J, Dracup K, Ezekowitz MD, et al. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation.* 2011;123(8):933-944. doi:10.1161/CIR.0b013e31820a55f5

10. Abbate A, Van Tassell BW, Biondi-Zoccai G, Kontos MC, Grizzard JD, Spillman DW, et al. Effects of interleukin-1 blockade with anakinra on adverse cardiac remodeling and heart failure after acute myocardial infarction [from the Virginia Commonwealth University-Anakinra Remodeling Trial (2) (VCU-ART2) pilot study]. *Am. J. Cardiol.* 2013;111(10):1394-1400. doi:10.1016/j.amjcard.2013.01.287

11. Abbate A, Kontos MC, Grizzard JD, Biondi-Zoccai GGL, Van Tassell B, Robati R, et al. Interleukin-1 blockade with Anakinra to prevent adverse cardiac remodeling after acute myocardial infarction (Virginia Commonwealth University Anakinra Remodeling Trial [VCU-ART] pilot study). *Am. J. Cardiol.* 2010;105:1371-1377. doi:10.1016/j.amjcard.2009.12.059

12. Butts B, Gary RA, Dunbar SB, Butler J. Methylation of Apoptosis-Associated Speck-Like Protein With a Caspase Recruitment Domain and Outcomes in Heart Failure. *J. Card. Fail.* Dec 14 2015. doi:10.1016/j.cardfail.2015.12.004

13. Puren AJ, Fantuzzi G, Dinarello CA. Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood

mononuclear cells and mouse spleen cells. *Proc. Natl. Acad. Sci. U. S. A.* Mar 2 1999;96(5):2256-2261.

14. Taniguchi S, Sagara J. Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC. *Seminars in Immunopathology.* 2007;29:231-238.

15. Yamamoto M, Yaginuma K, Tsutsui H, Sagara J, Guan X, Seki E, et al. ASC is essential for LPS-induced activation of procaspase-1 independently of TLR-associated signal adaptor molecules. *Genes Cells.* Nov 2004;9(11):1055-1067. doi:10.1111/j.1365-2443.2004.00789.x

16. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature.* Jul 8 2004;430(6996):213-218. doi:10.1038/nature02664

17. Toldo S, Mezzaroma E, O'Brien L, Marchetti C, Seropian IM, Voelkel NF, et al. Interleukin-18 mediates interleukin-1-induced cardiac dysfunction. *American journal of physiology. Heart and circulatory physiology.* Apr 1 2014;306(7):H1025-1031. doi:10.1152/ajpheart.00795.2013

18. Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, et al. Evidence for altered interleukin 18 (IL)-18 pathway in human heart failure. *FASEB J.* Nov 2004;18(14):1752-1754. doi:10.1096/fj.04-2426fje

19. Novick D, Kim SH, Fantuzzi G, Reznikov LL, Dinarello CA, Rubinstein M. Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity.* Jan 1999;10(1):127-136.

20. van Tassell B, Arena RA, Toldo S, Mezzaroma E, Azam T, Seropian IM, et al. Enhanced interleukin-1 activity contributes to exercise intolerance in patients with systolic heart failure. *PLoS ONE.* 2012;7(3):e33438. doi:10.1371/journal.pone.0033438

21. Van Tassell BW, Arena R, Biondi-Zoccai G, McNair Canada J, Oddi C, Abouzaki NA, et al. Effects of interleukin-1 blockade with anakinra on aerobic exercise capacity in patients with heart failure and preserved ejection fraction (from the D-HART pilot study). *Am. J. Cardiol.* Jan 15 2014;113(2):321-327. doi:10.1016/j.amjcard.2013.08.047

22. Nakajima K, Takeoka M, Mori M, Hashimoto S, Sakurai A, Nose H, et al. Exercise effects on methylation of ASC gene. *Int. J. Sports Med.* 2010;31:671-375. doi:10.1055/s-0029-1246140

23. Arena R, Cahalin LP, Borghi-Silva A, Phillips SA. Improving functional capacity in heart failure: the need for a multifaceted approach. *Curr. Opin. Cardiol.* Sep 2014;29(5):467-474. doi:10.1097/hco.0000000000000092

24. Bowen TS, Eisenkolb S, Werner S, Schwarzer M, Schuler G, Adams V. Inheriting a high aerobic fitness predisposes to skeletal muscle and endothelial dysfunction in chronic heart failure. *Int. J. Cardiol.* Jan 15 2016;203:353-356. doi:10.1016/j.ijcard.2015.10.125

25. Borlaug BA. The pathophysiology of heart failure with preserved ejection fraction. *Nat Rev Cardiol.* Sep 2014;11(9):507-515. doi:10.1038/nrcardio.2014.83

26. ElGuindy A, Yacoub MH. Heart failure with preserved ejection fraction. *Global cardiology science & practice.* 2012;2012(1):10. doi:10.5339/gcsp.2012.10

27. Luscher TF. Heart failure and left ventricular remodelling in HFrEF and HFpEF. *Eur. Heart J.* Feb 1 2016;37(5):423-424. doi:10.1093/eurheartj/ehw004

28. van Heerebeek L, Paulus WJ. Understanding heart failure with preserved ejection fraction: where are we today? *Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation.* Feb 24 2016. doi:10.1007/s12471-016-0810-1

29. Komajda M, Lam CS. Heart failure with preserved ejection fraction: a clinical dilemma. *Eur. Heart J.* Apr 2014;35(16):1022-1032. doi:10.1093/eurheartj/ehu067

30. Brown WM. Exercise-associated DNA methylation change in skeletal muscle and the importance of imprinted genes: a bioinformatics meta-analysis. *Br. J. Sports Med.* Dec 2015;49(24):1567-1578. doi:10.1136/bjsports-2014-094073

31. Denham J, O'Brien BJ, Marques FZ, Charchar FJ. Changes in the leukocyte methylome and its effect on cardiovascular-related genes after exercise. *Journal of applied physiology (Bethesda, Md. : 1985).* Feb 15 2015;118(4):475-488. doi:10.1152/japplphysiol.00878.2014

32. Kanzleiter T, Jahnert M, Schulze G, Selbig J, Hallahan N, Schwenk RW, et al. Exercise training alters DNA methylation patterns in genes related to muscle growth and differentiation in mice. *American journal of physiology. Endocrinology and metabolism.* May 15 2015;308(10):E912-920. doi:10.1152/ajpendo.00289.2014

33. Ronn T, Ling C. Effect of exercise on DNA methylation and metabolism in human adipose tissue and skeletal muscle. *Epigenomics.* Dec 2013;5(6):603-605. doi:10.2217/epi.13.61

34. Ronn T, Volkov P, Davegardh C, Dayeh T, Hall E, Olsson AH, et al. A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. *PLoS genetics.* Jun 2013;9(6):e1003572. doi:10.1371/journal.pgen.1003572

35. Zinovkina LA, Zinovkin RA. DNA Methylation, Mitochondria, and Programmed Aging. *Biochemistry (Mosc).* Dec 2015;80(12):1571-1577. doi:10.1134/s0006297915120044

36. Smith JA, Zagel AL, Sun YV, Dolinoy DC, Bielak LF, Peyser PA, et al. Epigenomic Indicators of Age in African Americans. *Hereditary genetics : current research.* Dec 2014;3(3). doi:10.4172/2161-1041.1000137

37. Ono T, Uehara Y, Kurishita A, Tawa R, Sakurai H. Biological significance of DNA methylation in the ageing process. *Age Ageing.* Jan 1993;22(1):S34-43.

38. Mays-Hoopes LL. DNA methylation in aging and cancer. *J. Gerontol.* Nov 1989;44(6):35-36.

39. Ebner N, Elsner S, Springer J, von Haehling S. Molecular mechanisms and treatment targets of muscle wasting and cachexia in heart failure: an overview. *Current opinion in supportive and palliative care.* Mar 2014;8(1):15-24. doi:10.1097/spc.0000000000000030

40. Jahng JW, Song E, Sweeney G. Crosstalk between the heart and peripheral organs in heart failure. *Exp. Mol. Med.* 2016;48:e217. doi:10.1038/emm.2016.20

41. Adams V, Spate U, Krankel N, Schulze PC, Linke A, Schuler G, et al. Nuclear factor-kappa B activation in skeletal muscle of patients with chronic heart failure: correlation with the expression of inducible nitric oxide synthase. *Eur J Cardiovasc Prev Rehabil.* Aug 2003;10(4):273-277. doi:10.1097/01.hjr.0000085250.65733.34

42. Gielen S, Adams V, Mobius-Winkler S, Linke A, Erbs S, Yu J, et al. Anti-inflammatory effects of exercise training in the skeletal muscle of patients with chronic heart failure. *J. Am. Coll. Cardiol.* Sep 3 2003;42(5):861-868.

43. Gary RA, Dunbar SB, Higgins MK, Musselman DL, Smith AL. Combined exercise and cognitive behavioral therapy improves outcomes in patients with heart failure. *J. Psychosom. Res.* Aug 2010;69(2):119-131. doi:10.1016/j.jpsychores.2010.01.013

44. Cipriano G, Jr., Cipriano VT, da Silva VZ, Cipriano GF, Chiappa GR, de Lima AC, et al. Aerobic exercise effect on prognostic markers for systolic heart failure patients: a systematic review and meta-analysis. *Heart Fail Rev.* Sep 2014;19(5):655-667. doi:10.1007/s10741-013-9407-6

45. Giallauria F, Lucci R, De Lorenzo A, D'Agostino M, Del Forno D, Vigorito C. Favourable effects of exercise training on N-terminal pro-brain natriuretic peptide plasma levels in elderly patients after acute myocardial infarction. *Age Ageing.* Nov 2006;35(6):601-607. doi:10.1093/ageing/afl098

46. Maria Sarullo F, Gristina T, Brusca I, Milia S, Raimondi R, Sajeva M, et al. Effect of physical training on exercise capacity, gas exchange and N-terminal pro-brain natriuretic peptide levels in patients with chronic heart failure. *Eur J*

Cardiovasc Prev Rehabil. Oct 2006;13(5):812-817. doi:10.1097/01.hjr.0000238396.42718.61

47. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* Oct 15 2013;62(16):e147-239. doi:10.1016/j.jacc.2013.05.019

48. Dharmarajan K, Hsieh AF, Kulkarni VT, Lin Z, Ross JS, Horwitz LI, et al. Trajectories of risk after hospitalization for heart failure, acute myocardial infarction, or pneumonia: retrospective cohort study. *BMJ.* 2015;350:h411. doi:10.1136/bmj.h411

49. Gary RA, Cress ME, Higgins MK, Smith AL, Dunbar SB. A combined aerobic and resistance exercise program improves physical functional performance in patients with heart failure: a pilot study. *Journal fo Cardiovascular Nursing.* 2012;27(5):418-430.

50. Gary RA, Cress ME, Higgins MK, Smith AL, Dunbar SB. Combined aerobic and resistance exercise program improves task performance in patients with heart failure. *Arch. Phys. Med. Rehabil.* Sep 2011;92(9):1371-1381. doi:10.1016/j.apmr.2011.02.022

Appendix A

Study Approval Documents

Nell Hodgson Woodruff School of Nursing, Emory University School of Medicine

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Title: **Feasibility of Exercise and Cognitive Retraining to Improve Memory, Attention and Concentration in Heart Failure**

Principal Investigator: **Rebecca Gary, RN, PhD, FAHA, FAAN**

Co-Investigators: **Felicia Goldstein, PhD; Elizabeth Corwin, RN, PhD; Kenneth Hepburn, PhD; Bryan Williams, PhD; Drenna Waldrop-Valverde, PhD**

Sponsor: **National Institute of Nursing Research**

Introduction

You are being asked to be in a medical research study and an optional sub-study discussed below. This form is designed to tell you everything you need to think about before you decide to consent (agree) to be in the study, the optional sub-study or not to be in either study. **It is entirely your choice. If you decide to take part, you can change your mind later on and withdraw from the research study.** The decision to join or not join the research study will not cause you to lose any medical benefits. If you decide not to take part in the main study or the optional substudy, your doctor will continue to treat you.

Before making your decision:

- Please carefully read this form or have it read to you
- Please listen to the study doctor or study staff explain the study to you
- Please ask questions about anything that is not clear

You can take a copy of this consent form, to keep. Feel free to take your time thinking about whether you would like to participate. You may wish to discuss your decision with family or friends. Do not sign this consent form unless you have had a chance to ask questions and get answers that make sense to you. By signing this form you will not give up any legal rights.

A description of this clinical trial (main study) will be available on www.ClinicalTrials.gov, as required by U.S. law. This website will not include

information that can identify you. At most the website will include a summary of the results. You may search this website at any time.

Main Study Overview

The **purpose** of this study is to compare 3 different groups to see which group is most effective for improving physical function, memory, thinking abilities and quality of life of people with heart failure. The study will be conducted on Emory University's campus and in your home for around 12 weeks and you will receive written educational information about your heart failure. You will be followed for an additional 12 weeks and the same measures will be taken again at 6 months.

Procedures

If you decide to take part in the study you will be asked to come to a special part of Emory University Hospital that provides a special space and staff for research studies, called the Clinical Integration Network (CIN). You will be asked to give a blood sample, answer questions, undergo a series of memory tests and a treadmill test. In addition, two sensors will be placed on each side of the temple area of your head for about 10 minutes to measure the oxygen level in your brain. All of these tests will take about 3 hours of your time. You will do these tests before you are assigned to your group and before the study intervention begins and again at 12 weeks. At about 6 months, the same questionnaires and the memory tests will be performed again which will take about 1.5 hours. You will not be asked to give a blood sample, undergo a treadmill test nor have the sensors placed on the temple area of your head at the 6 month appointment.

Your time in this study will last about 7 months. None of your appointments, medicines or the care you receive from your doctor will be changed based on your involvement in this research study.

You will be assigned to one of 3 groups to see which group is most effective for improving physical function, memory, thinking abilities and quality of life. Study group assignments are selected by a computer program; therefore you will not be able to choose the study group. These 3 groups are described below.

First Group (stretching and flexibility)

- You will be asked to do stretching and flexibility movements three times per week for 12 weeks for a minimum of 30 minutes.
- During the first 2 weeks of the stretching and flexibility program, a member of the research team will come to your home to show you how to perform the movements safely.
- After the first 2 weeks, a member of the research team will call you weekly to monitor your stretching and flexibility progress.
- It is expected that the stretching and flexibility movements will take about 90 to 120 minutes weekly during the study.
- You will receive heart failure educational materials that may be discussed during the course of the study.

Second Group (walking)

- You will be asked to walk 3 times per week for 12 weeks for a minimum of 30 minutes. As you progress throughout the study period your walking time and exertion level will increase as you become more conditioned.
- During the first 2 weeks of the walking program, a member of the research team will come to your home to walk with you to be sure you feel safe walking.
- You will be asked to use a heart rate (HR) monitor and a pedometer when you go walking. We will give you instructions on how to use this equipment.
- After the first 2 weeks, a member of the research team will call you weekly to monitor your walking progress and to change your walking duration or intensity level based on your progress.
- It is expected that the walking will take about 90 to 180 minutes weekly during the study.
- You will receive heart failure educational materials that may be discussed during the course of the study as well.

Third group (walking and computerized memory program)

- You will be asked to do the same walking routine as the second method and receive the same instructions as previous.
- In addition, you will be asked to take part in a computerized memory training program. You will receive instructions about the computerized memory training during the first 2 home visits along with the walking program.
- After the first 2 weeks, a member of the research team will call you weekly to monitor your progress in walking and in the computerized training program.
- It is expected that the combined walking and computerized memory program will take about 120 to 240 minutes weekly during the study.
- You will receive heart failure educational materials that may be discussed during the course of the study.

Overview of optional sub-study

The optional sub-study is no different than the main study you read about above. You will be assigned to one of three groups and will have the same procedures we described. You will be in the study for the same length of time. Your medical care will not be influenced by participating in the sub-study and your participation is completely voluntary. The only difference in the optional sub-study will be the collection of an additional tube of blood to examine how you respond to inflammation, cardiac proteins and gene expression. The results of the gene expression or other information from the optional study is not designed to make any sort of clinical diagnosis. These results will be placed in your medical record. You will not receive any results from these tests or interpretation of the study findings.

Testing

Testing will involve a visit to CIN at a time convenient for you and your family before you are assigned to one of the study groups. You will be asked to fill out several questionnaires that will take about 30 minutes or less of your time. Testing will also include several memory and thinking tests that will take about 75 minutes of your time. In addition, cerebral sensors will be placed against both sides of your head to measure the oxygen in your brain which will take about 10 minutes. You may request to stop and rest at any time during the testing procedures.

You will have about one teaspoon of blood taken from your arm. If you are in the optional sub-study you will have an additional teaspoon of blood drawn. The extra blood will not require an another needle stick. The blood test in the main study will be used to see how the walking or stretching and flexibility movements affect your memory and thinking abilities. The blood in the optional sub-study will be used to examine inflammation, cardiac function and gene expression related to your HF and exercise. Your blood will be coded, labeled and stored in a special freezer until it can be analyzed. When the study is completed, the blood sample in the main study and optional sub-study will be destroyed. Your blood sample will not be used for any other purpose than described here.

The treadmill test will measure your fitness level and takes 30 minutes or less to complete for most people. You will be asked to breathe in a mask while seated for about 2 minutes to measure your resting oxygen use. You will continue to wear the mask during the treadmill test. Before getting on the treadmill, your vital signs will be taken and you will be hooked up to a continuous electrocardiogram (EKG). You will be asked to walk on the treadmill for one minute to warm up. Once the treadmill test starts you will have your heart rate continuously recorded and monitored, your blood pressure will be taken every 5 minutes. You will walk at a constant rate of 3.2 mph during the treadmill test and a gradual incline every 2 minutes. You will be asked to continue to walk on the treadmill until you feel that you cannot walk any further.

After all of the testing has been completed, you will be assigned to one of the study groups. You will receive individualized instruction depending on which group you are in during the 2 home visits.

Risks

The most common risks during the treadmill tests are:

- becoming short winded or tired,

Less commonly, you may experience:

- have a rapid or irregular pulse,
- have high or low blood pressure changes,

Rarely, you may experience the following:

- develop chest pain or feel lightheaded,
- you could fall while performing the treadmill test
- experience bone or muscle pain which is also rare.

In addition, it is possible that if you have an internal cardiac device:

- it could fire which is rare and you could fall as a result

The treadmill test will be administered in the hospital by an exercise specialist and under the supervision of a cardiologist. In addition, if an adverse event should occur, nurses, doctors and emergency equipment are available nearby to help you which rarely occurs. A continuous electrocardiogram (EKG) will be taken. If there are any abnormal EKG changes during the treadmill test, it will be stopped right away. If there are any negative vital sign changes such as lower or higher BP, irregular heartbeats, chest pain or dizziness the study cardiologist (Dr. Butler) will be notified. If treatment is necessary you will receive emergency treatment in the CIN and transferred to the ER for further evaluation, which rarely occurs.

Walking-related risks

The risks of the walking program are expected to be minimal. Any potential cardiovascular (CV) event that poses risk to you should be detected during the treadmill test. In addition, the walking time will be limited to 30 minutes during the first 2 weeks and at an intensity level that is not likely to result in any heart problems; if necessary you can rest as much needed until the 30 minute duration of walking is completed.

You will be asked to wear a special Polar Heart Rate (HR) monitor so that HR and exertion level can be closely monitored. A research team member will walk with you at home 2 times to better ensure your safety prior to walking at home unsupervised; the risk associated when you are walking alone therefore, is anticipated to be very minimal. You will be provided with detailed directions on how to self-monitor your heart rate, blood pressure and symptoms associated before, during and after walking.

Also, you will be given a target heart rate range to stay within during the study period. You will be asked to wear the Polar HR monitor during each walking session. You will be asked to take your heart rate, blood pressure (if machine available) and weight prior to and after each walking session and record it in your walking calendar. You will be asked to call the research nurse if your heart rate or blood pressure is outside your normal range. If you are having symptoms, experience increased shortness of breath or have a greater than 2 pound weight gain over the previous 24 hours, you will be instructed not to walk. You will be asked to take your medicines as usual prior to walking.

You will be shown how to wear the Polar heart rate monitor by a research nurse. When your heart rate approaches 5-10 beats of the target heart rate range you will be asked to slow the walking pace down. If you have an ICD, you will be asked to keep your heart rate 15 beats below the firing range at all times. In addition, you will be instructed to monitor your rate of perceived exertion (RPE), or how hard you feel you are working during the walking session. You will be given instructions on how to monitor your rate

of perceived exertion (RPE) using the Borg 6 to 20 scale, and to keep your RPE at 12-13 during the initial weeks and to gradually progress with instructions to 15 as directed.

For safety reasons, you will be asked to carry a cell phone when you walk at home in the event of an emergency or sudden event. If you have had a heart attack before or prescribed nitroglycerin (NTG) by your doctor you will be asked to carry your NTG with you during each walk. If chest pain occurs during walking, you will be asked to stop exercising and to take a NTG as directed. If chest pain continues or you become increasingly short winded, you will be provided with specific actions to take during the first home visit. If an ICD fires, you will be instructed to call 911 if any symptoms are present or to notify your cardiologist if no symptoms for possible evaluation.

Other Study-Related Risks

For some people answering questions or filling out questionnaires can be emotionally distressing. If that happens, a counselor will be made available. One of our questionnaires will ask you about depressive symptoms. If you indicate you are having symptoms of depression from the responses on the questionnaire, mental health referral information will be provided and you will be asked if you would like a research team member to assist you in setting up a referral. If at any time you indicate serious depressive symptoms or intent to hurt yourself, a family member will be informed and/or their primary care provider (or cardiologist) alerted.

The risk to you if you are in the memory training program is anticipated to be minimal. You will be taught how to use the memory training program on a computer by a research nurse. It is possible that some participants in the study may have other stress or anxiety related to being in this program. The 2 home visits that will be used to review the memory training program are expected to lower any unusual stress or concern about being in the study. In addition, the research nurse will be contacting you on a weekly basis to discuss any concerns or issues related to memory training program from weeks 3-12. If you are in the stretching/flexibility movement group the potential risk is also expected to be minimal. You will be taught how to use stretching and flexibility movements by a research nurse.

There are no additional risks if you participate in the optional sub-study in relation to findings. The results of the gene expression would not place you or your family at any greater risks if confidentiality were breached for some reason than the results from the main study.

Blood Draw-Related Risks

The blood sample in the main study and the optional sub-study will be collected by a research nurse or laboratory assistant trained in phlebotomy techniques in the CIN before the study begins and again at 3 months. No additional needle sticks are required should you decide to participate in the optional sub-study. Slight bruising at the site of the needle stick is possible. You may also feel dizzy or faint when your blood is drawn. If

you are on blood thinner medications, there will be additional pressure to the site until it stops bleeding.

Benefits

The benefits to you for taking part in this study may be that you have better memory and thinking abilities, better abilities to perform daily activities and quality of life. What is learned from this study may be useful to other people with similar heart conditions and memory issues who are experiencing symptoms related to performing daily activities or managing their affairs. Doctors and nurses may also benefit from the information in this study by learning how to manage patients with similar heart conditions who have memory concerns and reduced physical function. While the study is designed to benefit you it is possible there will be no benefit from being in this study.

Alternatives

You do not have to take part in the study in order to receive treatment. Other procedures/treatments that you have been receiving or are currently receiving from your doctor are the usual care for your heart condition.

Confidentiality

Certain offices and people other than the researchers may look at your medical charts and study records. Government agencies and Emory Health System employees overseeing proper study conduct may look at your study records. These offices include the Office for Human Research Protections, the Emory Institutional Review Board, , the Emory Office of Research Compliance, the Office for Clinical Research, the Clinical Trials Audit & Compliance Office, the Radiation Safety Committee, etc. Study sponsors may also look at your study records. Emory Health System will keep any research records we create private to the extent we are required to do so by law. A study number rather than your name will be used on study records wherever possible. Your name and other facts that might point to you will not appear when we present this study or publish its results.

Research Information Will Go Into the Medical Record

If you are or have been an Emory Health System patient, you have an Emory Health System medical record. If you are not and have never been an Emory or Grady Health System patient, you do not have one. Please note that an Emory medical record **will** be created if you have any services or procedures done by an Emory Health System provider or facility for this study.

If you agree to be in this study, a copy of the consent form and HIPAA patient form that you sign **will** be placed in your Emory Health System medical record. Emory Health System may create study information about you that can help Emory Healthcare take care of you. For example, the results of study tests or procedures. These useful study results **will** be placed in your Emory Health System medical record. Anyone who has access to

your medical record will be able to have access to all the study information placed there, including results form the optional sub-study. The confidentiality of the study information in your medical record will be protected by laws like the HIPAA Privacy Rule. On the other hand, some state and federal laws and rules may not protect the research information from disclosure.

Emory Health System do not control results from tests and procedures done at other places, so these results would not be placed in your Emory Health System medical record. They will not likely be available to Emory Health System to help take care of you. Emory Health System also do not have control over any other medical records that you may have with other healthcare providers. Emory Health System will not send any test or procedure results from the study to these providers. If you decide to be in this study, it is up to you to let them know.

Compensation/Costs

You will be paid $50.00 each time you complete one of the testing time points. If you stay in the study, you will receive a total of $150.00. You will not be paid for the home visits or telephone calls. There will be no costs to you for taking part in the study. All study tests are free. We will provide money to cover transportation and parking costs associated with laboratory, memory testing and treadmill tests at the CIN

In case of injury:
If you get ill or injured from being in the study, Emory would help you to get medical treatment. Emory and the sponsor have not, however, set aside any money to pay you or to pay for this medical treatment. The only exception is if it is proved that your injury or illness is directly caused by the negligence of an Emory or sponsor employee. "Negligence" is the failure to follow a standard duty of care.

If you become ill or injured from being in this study, your insurer will be billed for your treatment costs. If you do not have insurance, or if your insurer does not pay, then you will have to pay these costs.

If you believe you have become ill or injured from this research, you should contact Dr. Gary at telephone number 404-727-8360. You should also let any health care provider who treats you know that you are in a research study.

Voluntary participation/Withdrawal
Your participation in this study is completely voluntary and you have the right to refuse to be in this study. You can stop at any time after giving your consent. This decision will not affect in any way your current or future medical care or any other benefits to

which you are otherwise entitled. You will be provided with a copy of this consent form to keep.

The study investigators may stop you from participating in this study at any time if they decide it is in your best interest, or if you do not follow study instructions.

Contact Persons
If you have any questions about this study call Dr. Rebecca Gary at (404) 727-8360. If you have any questions about your rights as a participant in this study, contact the Emory University Institutional Review Board at 404-712-0720 or toll-free at 1-877-503-9797 or irb@emory.edu.

New Information
It is possible that the researchers will learn something new during the study about the risks of being in it. If this happens, they will tell you about it. Then you can decide if you want to continue to be in this study or not. You may be asked to sign a new consent form that includes the new information if you decide to stay in the study.

Entitlement to copy
You will receive a copy of this consent form to keep for your records. If you are interested what the study findings are after the results are in and analyzed, the findings will be available to you.

If you are willing to volunteer for research in the main study, please sign below.

_____	_____	_____
Participant's name (printed)	**Date**	**Time**

_____	_____	_____
Participant's name (signature)	**Date**	**Time**

_____	_____	_____
Person Obtaining Consent	**Date**	**Time**

If you are willing to volunteer for research in the optional sub-study please sign below:

_____

Participant's name (printed) Date Time

_____

Participant's name (signature) Date Time

_____

Person obtaining Consent Date Time

EMORY UNIVERSITY | Environmental Health and Safety Office | Research Health & Safety Committee
Research Administration | Research/ Biological Safety

October 19, 2015

Brittany Butts, PhD Student
Dept. of Academic Advancement
655 Whitehead Building

Biosafety File #: **H10-169-15**

Proposal Title: **Inflammasome pathways in heart failure**

Personnel working on this project: ***Brittany Butts***

This approval is valid until October 1, 2016.

Dear Dr. Butts:

The proposal to work with biological toxins, infectious agents, and/or recombinant DNA molecules referenced above was reviewed and approved by the Research Health and Safety Committee (RHSC).

The attached addendum outlines the specific agents that you are approved to work with in this protocol and lists specific requirements that must be maintained.

NIH Guidelines rDNA Classification: n/a

For any additions or changes to the protocol, an amendment form needs to be submitted. The amendment form is available at www.ehso.emory.edu.

The Biosafety assigned file number **H10-169-15** needs to be included in all correspondence.

You must complete an Updated Biosafety Form by September 10, 2016. Three months prior to the expiration date, you will receive a memorandum requesting you to indicate your interest in continuing work on the protocol for an additional year. Upon receipt and after review of a completed update form, completed training dates and any occupational health requirements, the protocol will be renewed for one year.

Gary W. Miller, Ph.D.
Chair, Research Health and Safety Committee

Professor and Associate Dean for Research
Department of Environmental Health
Rollins School of Public Health

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Appendix B

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