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Methylation of Apoptosis-Associated Speck-Like Protein With a Caspase Recruitment Domain and Outcomes in Heart Failure

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Abstract

Background—Heart failure (HF) is associated with inflammation characterized by the formation of the inflammasome, which triggers maturation of inflammatory cytokines. ASC, a vital component of the inflammasome, is controlled through epigenetic modification, which may be a candidate pathway for worsening HF. This study examined the inflammasome pathway in HF and the relationships between ASC CpG methylation and outcomes in HF.

Methods and Results—Stored samples from 155 HF outpatients (ejection fraction 29.9 ± 14.9) were analyzed for % methylation of seven CpG sites in the intron region preceding exon-1 of the ASC gene. ASC methylation was inversely related to ASC mRNA ($r = -.33, P < .001$) and protein ($r = -.464, P < .001$). 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.

Conclusion—Increased methylation of CpG sites in the intron region of ASC is associated with improved outcomes in HF. The associated decrease in ASC expression implicates this inflammatory mediator as a possible driver of HF outcomes and may represent a therapeutic target.

Keywords

heart failure; epigenetic; inflammation

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INTRODUCTION

Despite advances in therapies for HF with reduced ejection fraction (HFrEF), these patients remain at high risk. There are no known therapies for HF patients with preserved EF (HFpEF). Once hospitalized, these patients have a ~30% risk of dying within a year, irrespective of EF.¹ Physical disability in HF is a major reason for anxiety, depression, and poor quality of life (QoL).^{2,3} Thus there remains a pressing need to investigate novel pathways of HF progression to facilitate development of new therapies.

HF is associated with a low-grade inflammation that leads to cardiac remodeling.⁴ This is initiated by host-derived molecules, danger-associated molecular patterns (DAMPs), that modulate fibrosis, apoptosis, and hypertrophy.⁵⁻⁷ The inflammatory response amplifies DAMP production in a positive-feedback loop, accelerating these pathological processes.⁸ Poor cardiac function triggers the release of cytokines, such as tumor necrosis factor alpha (TNF α), which magnify inflammation and remodeling.^{4,6,9,10} DAMP-activated inflammation occurs via the inflammasome, a complex of intracellular proteins that recognizes DAMPs and triggers maturation of cytokines to initiate and amplify inflammation.^{7,11,12} 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.^{10,13,14} The activated inflammasome cleaves pro-caspase-1 into caspase-1,^{13,15} which in turn, activates interleukin (IL)-1 family inflammatory cytokines IL-1 β and IL-18, by cleaving pro-IL-1 β and pro-IL-18 into active forms.^{16,17} Thus, the inflammasome is a powerful mediator of the immune response via caspase-1 activation of IL-1 β and IL-18.

ASC recruits pro-caspase-1 to the inflammasome complex and is necessary for activation of pro-caspase-1 into caspase-1.^{4,5,17,18} ASC expression is controlled by epigenetic modification via methylation of a CpG island in the promoter region of exon 1¹⁹⁻²². DNA methylation of ASC represses recruitment of RNA polymerase II to the promoter region and leads to transcriptional silencing.²³ Hypermethylation of the ASC CpG island leads to gene silencing and inhibition of apoptosis. Suppression of ASC expression has been shown to decrease IL-1 β activation and may be an inhibitor of inflammasome-mediated inflammation.²⁴

Global methylation studies show varying DNA methylation with age,²⁵⁻²⁷ exercise,^{28,29} diet,³⁰⁻³² and environmental factors.³³⁻³⁵ While NLRP3 and caspase-1 have inflammatory functions independent of the inflammasome, ASC functions only as an adapter protein as part of the inflammasome; the level of CpG methylation of ASC may control inflammasome formation.³⁶ Thus, inflammasome formation and activation may be reduced via increased ASC methylation. While the role of ASC methylation in HF is not known, we theorize that decreased ASC expression, related to increased ASC methylation, is associated with decreased inflammasome-mediated inflammation. The purpose of this study is to examine the inflammasome pathway in HF and the relationships between ASC CpG methylation and physical, psychosocial, and QOL outcomes in HF.

METHODS

Study population

Stored blood samples (N=155) from a prospective cohort study that enrolled outpatients with HF from two university-affiliated hospitals were used. Inclusion criteria included age >18 years with a diagnosis of HFrEF or HFpEF. The diagnosis of HFpEF required, in addition to clinical diagnosis, elevated B-type natriuretic peptide level >200 pg/dl and/or echocardiographic evidence of diastolic dysfunction. Exclusion criteria included congenital heart disease, heart transplantation, cardiac infiltrative disease (e.g., amyloidosis), other solid organ transplantation, cancer, and continuous inotrope infusion. *Charlson comorbidity index* (CCI) was used to control for comorbidities.³⁷ All measures in this analysis were collected at a baseline visit.

ASC Methylation and Expression

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs). DNA (1 µg) was treated with sodium bisulfite using a commercial kit. Bisulfite-modified DNA was amplified by PCR followed by pyrosequencing for methylation quantification according to Nakajima et al.³⁸ Methylation of 7 CpG sites in the promoter region of exon 1 were measured³⁸ and analyzed as mean % methylation. The mean % methylation among the 7 CpG sites for each individual was calculated as the sum of % methylation of the CpG sites divided by 7.

250 ng of total RNA were reverse transcribed using the high-capacity cDNA reverse transcription kit from Life Technologies®. Each 20 µL reaction contained 1X reaction buffer, the addition of 1.5 mM MgCl₂, 4mM dNTP mix, 1X random primer mix, 50 U of MµLtiScribe® Reverse Transcriptase, and 20 U of RNase Inhibitor. The samples were incubated at 25°C for 10 min, followed by a 37°C for 2hrs, and a final 85°C incubation for 5 min. Samples were stored at 4°C until expression analysis. Samples were diluted 1:8 prior to expression analysis. Each 20µL reaction contained 1X PCR buffer at 1.5 mM MgCl₂, the addition of 3.5 mM MgCl₂, 200µM dNTP, 0.6 U of Taq DNA Polymerase, 1X GAPDH VIC labeled Taqman® assay, and 1X FAM labeled gene specific Taqman® assay. PCR was carried out in a Qiagen Roto-Gene Q instrument with a 72-sample rotor. An initial denaturation of 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min was used for the real-time PCR parameters. EpigenDx, Hopkinton, MA was used to perform ASC expression analysis. ASC mRNA expression is presented as the ratio of ASC expression to GAPDH expression.

Plasma IL-1β, IL-18 and ASC were measured in duplicate using human ELISA kits. Plates were read on a Molecular Devices ELISA plate reader. Curve fitting was selected among linear, quadratic and 4-point based on the best regression coefficient using SoftPro.

Outcomes

The six-minute walk test (6MWT) was used to measure functional ability.³⁹ Participants were instructed to walk as far as possible for six minutes and the distance was measured in meters. Depressive symptoms were measured using the Patient Health Questionnaire

(PHQ-9), a 9-item depression scale representing the DSM-IV symptoms.⁴⁰ Total score can range from 0 to 27, with higher scores representing greater symptoms. The Generalized Anxiety Disorder Assessment (GAD-7) is a self-report questionnaire that measures the presence and severity of anxiety, and is comprised of 7 questions with scores ranging from 0 to 21. Total scores of 5, 10, and 15 are cutoffs for mild, moderate, and severe anxiety, respectively.⁴¹ The Kansas City Cardiomyopathy Questionnaire (KCCQ)⁴² is a 23-item questionnaire that quantifies physical function, symptoms, social function, and health-related QoL in HF.⁴³ An overall summary score was derived from the physical function, symptom (frequency and severity), social function, and QoL domains. Scores are transformed to a range of 0–100 with higher scores reflecting better health status.

Participants were followed prospectively for up to 47 months from the initial baseline visit to collect clinical event data. Clinical events were comprised of all cause hospitalization, all cause death, heart transplant, or ventricular assistive device [LVAD]) implantation.

Statistical Analysis

Data were reviewed for normality assumptions and outliers. No data points were excluded from analysis due to outlier status. Pearson correlation analysis was used to examine the direction and strength of the relationship between ASC methylation and IL-1 β and IL-18. Student t-tests for normal distributions and Mann Whitney tests for non-parametric distributions were used to examine differences in ASC methylation and cytokines among the categories of outcomes. Linear regression and partial correlation analysis was used to examine relationships between ASC methylation and outcomes, adjusting for EF, age and CCI. Cox regression modeling was used to examine the relationships between ASC methylation and expression and clinical events. Covariates included in the model were chosen based on previously established associations with HF hospitalization and/or death and were collected at the baseline time point. The covariates were included in the model with forced entry of all variables simultaneously. All data were analyzed using SPSS version 22.0.

RESULTS

Baseline Patient Characteristics

Baseline data are presented in Table 1. Most participants were male and 47% were African American. EF ranged from 8 to 65%, with 76% of the participants had an EF <40%. BNP was highly skewed (skewness statistic 3.5 \pm 0.2) and was log transformed for all analyses.

ASC methylation and expression

Increased % methylation of 7 CpG sites preceding exon 1 of ASC was related to decreased ASC mRNA and protein expression (Figure 1). Decreased ASC expression was related to decreased IL-1 β expression (r =.619, P <.001). Decreased IL-1 β was also related to increased ASC methylation (r =-.24, P =.005). No significant relationship was found between ASC methylation or ASC expression and IL-18 expression.

Mean % ASC methylation ranged from 4.6 to 6.8 (Table 2), and was not associated with age ($r=-.09$, $P=.33$), gender ($r=.03$, $P=.13$), or race ($r=-.05$, $P=.56$). Body mass index had a weak negative association with mean % ASC methylation ($r=-.169$, $P=.053$). ASC methylation was not correlated with comorbidities, including hypertension ($r=-.003$, $P=.97$), diabetes ($r=-.05$, $P=.60$), chronic pulmonary disease ($r=.03$, $P=.77$), and renal disease ($r=-.13$, $P=.15$), or with CCI ($r=-.037$, $P=.683$). When added as covariates in linear and logistic models, they had no impact on the relationships between ASC methylation and ASC expression, cytokine expression, and outcomes.

ASC Methylation and Outcomes

Median 6MWT distance was 352 meters, with 32% ($N=42$) of participants walking <300 meters. PHQ-9 scores ranged from 0 to 25, with 27% ($N=39$) scoring ≥ 10 (moderate or severe depression). GAD-7 scores ranged from 0 to 21, with 16% ($N=25$) meeting the criteria for anxiety with scores ≥ 10 . The median KCCQ overall summary score was 70.3, with scores ranging from 12.8 to 100. Outcome data are presented in Table 3.

Mean % ASC methylation had a positive linear relationship with EF with a plateau around 40% (Figure 2). Higher mean % ASC methylation was positively associated with 6MWT and KCCQ summary score and negatively associated with PHQ-9 and GAD-7 total scores.

Participants with HF_rEF had less ASC methylation than those with HF_pEF (5.77 vs. 6.48, respectively; Mann-Whitney U test $z=-8.0$, $P<0.001$). In addition, IL-1 β and IL-18 were higher in participants with HF_rEF than those with HF_pEF (IL-1 β : 1.90 vs 1.57, respectively; $t=-2.19$, $P=.03$), [IL-18 319.93 vs 293.92, respectively; $t=-2.21$, $P=.029$]. % mean ASC methylation decreased with increase in New York Heart Association functional class (6.33 Class I, 5.73 class III, $F=6.4$, $P=0.002$). 6MWT distance of <300 meters is associated with mortality risk in HF.⁴⁴ Participants who walked <300 meters had lower ASC methylation than those who walked >300 meters (5.82 ± 0.54 vs. 6.09 ± 0.44 , respectively; $t=2.89$, $P=0.005$). Participants with moderate or severe depressive symptoms or anxiety had lower ASC methylation. Mean % ASC methylation for participants with a total PHQ-9 ≥ 10 , indicative of moderate or severe depressive symptoms,⁴⁵ was 5.5 ± 0.39 and 6.1 ± 0.45 for those who scored < 10; $t=6.58$, $p<.001$. Mean % ASC methylation was 5.42 ± 0.46 for participants with a total GAD-7 score ≥ 10 , indicative of moderate or severe anxiety,⁴¹ and 6.06 ± 0.44 for those who scored < 10; $t=5.9$, $p<.001$.

Overall 110 (71%) participants experienced a clinical event (88 had at least one hospitalization, 16 ventricular assist device implants, 3 deaths, and 3 heart transplants). Among participants experiencing an event, time to clinical event ranged from 2 days to 45 months (mean time 10.5 ± 10.9 months). Multivariable Cox regression analysis for risk of clinical event, adjusting for age, LVEF, and CCI, was performed (Table 4). A 6MWT distance of < 300 meters was associated with a higher risk of experiencing a clinical event ($HR=3.4$, $P=.001$). ASC methylation was associated with a lower risk of a clinical event ($HR=0.16$, $P=.025$), while ASC protein ($HR=1.28$, $P=.005$) and mRNA ($HR=1.003$, $P=.045$) expression were associated with a higher risk of a clinical event.

DISCUSSION

This study is the first to examine ASC CpG methylation and expression in HF patients. Increased methylation of 7 CpG sites surrounding exon 1 was associated with decreased ASC expression. In addition, increased CpG methylation of ASC was associated with decreased plasma IL-1 β . There was no relationship between ASC methylation and plasma IL-18, which was unexpected. Although active inflammasome has been shown to activate both IL-1 β and IL-18, these circulating cytokines may have resulted from redundant pathways of inflammation.

Mean % ASC CpG methylation was 5.96 \pm 0.5%, which is higher than healthy elderly but lower than younger adults, as reported by Nakajima et al.³⁸ Although they found a difference in levels of ASC methylation between the younger and older groups, this study found no association with age in HF. Previous studies have established that there is a decrease in global DNA methylation with age,²⁵⁻²⁷ but this relationship has not been established in HF. The age-related changes in healthy adults are likely not relevant in chronic HF. The physiological and psychological stressors of chronic disease can lead to premature cellular aging,⁴⁶ which may be reflected by DNA methylation. Additional studies are necessary to determine the relationships between epigenetic control of gene expression and age in HF.

ASC methylation had a curvilinear relationship with EF with a strong positive linear relationship that begins to plateau around 40%. There may be an upper limit to the amount of ASC CpG methylation in HF. If so, this limit is just under 7%, which is in line with the group of healthy, young adults,³⁸ indicating this may be the upper limit for the non-cancer population. There was a significant difference in ASC methylation between HF_rEF and HF_pEF, which may reflect the different pathophysiologies of these two conditions and require further investigation.

ASC methylation was associated with better psychosocial and QoL status. Inflammatory processes have been linked to depression and QoL. Depression and poor health status may potentiate the positive feedback loop of inflammation implicated in HF pathology. CpG demethylation may result from chronic inflammation, and the processes of ASC methylation and psychosocial and QoL status may be interrelated. Further animal model and human studies aimed at reducing physiological and psychosocial stress may help elucidate these mechanisms.

Both ASC methylation and ASC expression were associated with clinical events. Higher ASC methylation was associated with a decreased risk of hospitalization or death occurring, while ASC expression was associated with an increased risk of a clinical event. These results suggest that inflammasome is potentially integral component of pathophysiological processes in HF, requiring further examination of the mechanisms controlling inflammasome formation and activation. ASC is an adaptor protein with an N-terminal pyrin domain that links a pattern recognition receptor (such as NLRP3) with caspase-1 via a C-terminal caspase-recruitment domain. Once constructed and activated, the inflammasome functions to sense exogenous and endogenous danger signals and to initiate inflammatory or

apoptotic pathways. Suppression of ASC expression leads to decreased IL-1 β processing,²⁴ implicating control of ASC expression as an inhibitor of inflammasome activation and the downstream caspase-1 dependent inflammatory pathway. Therefore, epigenetic modulation of ASC expression likely plays a vital role in the regulation of inflammation

Previous work examining ASC CpG methylation has been in cancer studies assessing the link between ASC, apoptosis, and carcinogenesis.^{19,20,22} These studies approached epigenetic control of ASC as a dichotomous on/off switch in cancer cells^{19,22,47,48} where hypermethylation (off) is aberrant, leading to a faulty switch in the tumor suppressor machinery. Here we approach ASC CpG methylation as a regulator of inflammation, in which individuals have varying levels of methylation according to yet to be discovered physiological processes. Where in cancer CpG methylation is excessive or low, in chronic disease ASC expression may be optimized under complex epigenetic regulation. Previous studies have demonstrated varying levels of CpG methylation,³⁸ however, few studies linked DNA methylation status with protein expression and subsequent physiological pathways in chronic disease. We demonstrated a positive association between ASC CpG methylation and ASC gene expression. Further, ASC CpG methylation was negatively associated with plasma IL-1 β . These results suggest that level of DNA methylation is an important mechanism controlling ASC gene expression and may lead to decreased inflammasome activation. Further studies examining the direct relationships along the inflammasome activation pathway are needed to untangle the associations between chronic inflammation and HF pathophysiology. Given these results, ASC CpG methylation may prove to be a primary regulator of the pathogenesis of chronic inflammatory diseases such as HF.

In this cross-sectional study, we were not able to assess changes in DNA methylation over time or after an intervention. Further, we are not able to assess if changes in DNA methylation are related to changes in ASC expression. PBMCs are useful to measure systemic inflammation, however examining the processes in cardiac tissue could shed further light on the relationships between inflammasome and remodeling. Inflammasome activation can occur in many tissues and has been implicated in several chronic diseases.^{49–53} Considering the multiple co-morbidities of this sample of HF patients, inflammasome activation in other tissues may contribute to circulating levels of inflammatory proteins. In addition, inflammasome activation in tissues other than the heart and PBMCs may contribute to the pathophysiology of HF.

This study examined DNA methylation and did not focus on other epigenetic processes. Methylation of H3K9 is a common site of transcriptional repression,⁵⁴ and methylation of H3K9 is commonly linked to methylation of nearby CpG sites.^{55,56} Although the methylation status of histone lysines and CpG sites are often mechanistically linked, the methylation of histones are more readily reversible and may be a better measure of immediate transcriptional control. Conversely, the methylation status of CpG islands are indicative of more long-term control and may prove to be a better marker of the pathophysiological status in chronic disease.

In conclusion, increased methylation of CpG sites in the intron region of ASC is associated with improved physical, psychological and QoL outcomes in HF. The associated decrease in

ASC expression implicates this inflammatory mediator as a possible driver of HF outcomes, providing an avenue to explore novel interventions.

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Abbreviations list

ASC	apoptosis-associated speck-like protein with a caspase recruitment domain
KCCQ	Kansas City Cardiomyopathy Questionnaire
PHQ-9	Patient Health Questionnaire
GAD-7	Generalized Anxiety Disorder 7-item scale
IL	interleukin

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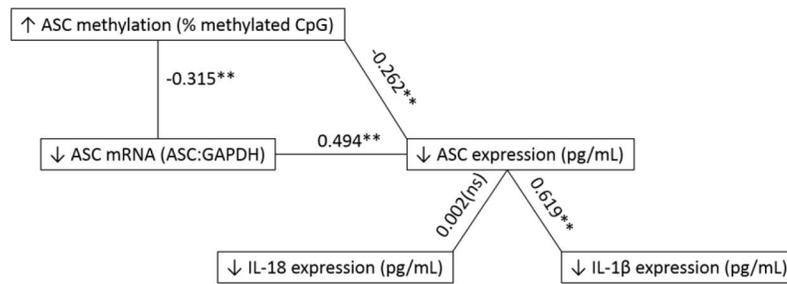
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Highlights

1. Heart failure (HF) is associated with inflammation characterized by the formation the inflammasome, which triggers maturation of inflammatory cytokines.
2. ASC, a vital component of the inflammasome, is controlled through epigenetic modification, which may be a candidate pathway for worsening HF.
3. ASC methylation in this study was inversely related to ASC mRNA and protein.
4. ASC methylation had a positive linear relationship with ejection fraction, quality of life, and six-minute walk test, and a negative linear relationship with depression and anxiety.
5. Higher ASC methylation was associated with a lower risk for clinical adverse events while higher protein and mRNA expression were associated with a greater risk.



Pearson's r values listed

**Correlation is significant at the 0.01 level (two-tailed)

Figure 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-1 β expression. No significant relationship was found between ASC expression and IL-18 expression.

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

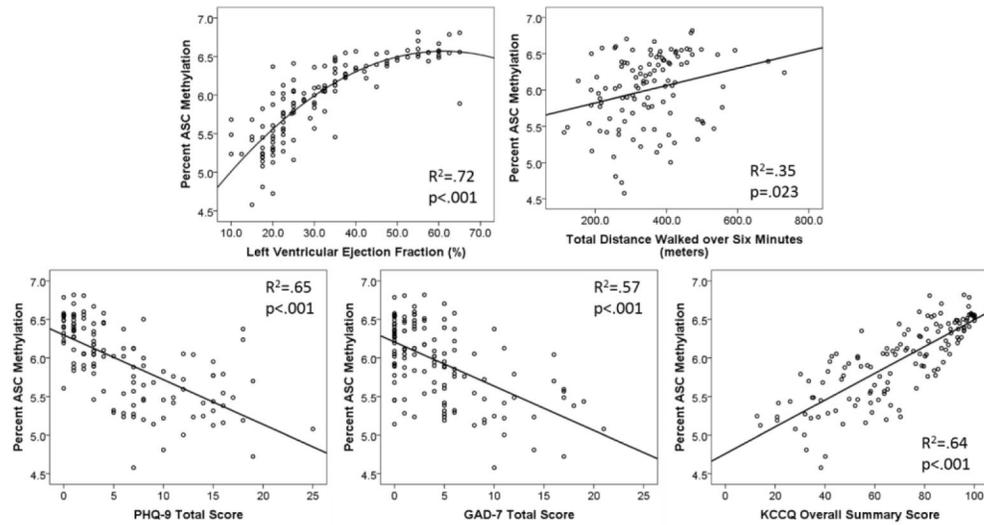


Figure 2. Linear relationships between percent ASC Methylation and heart failure outcomes
 Left ventricular ejection fraction (LVEF) and LVEF-squared were added as covariates in the other linear models.

PHQ-9 – Patient Health Questionnaire, GAD-7 – Generalized Anxiety Disorder, KCCQ – Kansas City Cardiomyopathy Questionnaire

Table 1

Sample Demographics and Clinical Characteristics

N=155	Range	Mean (SD)	Median (IQR)
Age (years)	27 – 87	56.9 (12.0)	57 (50–64)
BMI (kg/m ²)	17.2 – 52.3	30.8 (7.3)	29.7 (25.3–30.1)
LVEF (%)	7.5 – 65	29.9 (14.9)	25 (20–37.5)
Charlson Comorbidity Index	1 – 10	4.38 (2.15)	4 (3–6)
Systolic Blood Pressure (mmHg)	72 – 223	114.1 (22.8)	110 (100–26)
Sodium (mEq/L)	129 – 145	138.3 (2.9)	138 (136–140)
BNP (pg/mL)	6 – 4612	469.6 (680.1)	226 (68–638)

		N	%
Gender	Male	99	63.9%
	Female	56	36.1%
Race	Black	73	47.1%
	White	81	52.3%
	Other	1	0.6%
	I	11	7.2%
NYHA class	II	105	68.6%
	III	37	24.2%
	IV	0	N/A
Type of HF	HFrEF	118	76.1%
	HFpEF	37	23.9%

BMI: Body mass index, LVEF: Left ventricular ejection fraction, BNP: brain natriuretic peptide, NYHA: New York Heart Association, HF: Heart Failure, HFrEF: HF and reduced ejection fraction (<40%), HFpEF: HF and preserved ejection fraction (≥40%)

Table 2

ASC and IL-1 Cytokines

N=155	Mean \pm SD	Range
Mean ASC Methylation (%)	5.96 \pm 0.5	4.6 – 6.8
ASC:GAPDH mRNA	1.15 \pm 0.07	0.91 – 1.41
ASC protein (pg/mL)	4.31 \pm 2.7	0.27 – 15.82
IL-1 β (pg/mL)	1.81 \pm 0.8	0 – 3.5
IL-18(pg/mL)	313.31 \pm 62.9	109.2 – 452.3

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

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Table 3

Six-Minute Walk Test, Psychological, and Quality of Life Measures

N=155	Range	Mean (SD)	Median (IQR)
6-minute walk test (meters)	112.7–731.5	353.8 (108.6)	351 (276–416)
Patient Health Questionnaire-9	0–25	3.61 (5.9)	4 (1–10)
Generalized Anxiety Disorder -7	0–21	4.44 (5.2)	3 (0–6)
Kansas City Cardiomyopathy Questionnaire	12.8–100	67.31 (23.7)	70 (49.6–90.2)

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Table 4

Multivariable Cox Regression Analysis for Risk of Clinical Event Adjusted for Covariates

Variable	Hazard Ratio	95% Confidence Interval	p-value
6 minute walk distance <300m	3.4	1.68 – 7.26	.001
ASC % methylation	0.16	0.03 – 0.79	.025
ASC protein expression (pg/mL)	1.28	1.11 – 1.39	.005
ASC mRNA expression (ASC:GAPDH)	1.003	1.00 – 1.010	.045

Hazard ratios adjusted for age, left ventricular ejection fraction, and comorbidities

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