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# Protein Cytokines, Cytokine Gene Polymorphisms, and Potential Acute Coronary Syndrome Symptoms

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**Abstract**

The purpose of this study was to determine whether relationships exist among protein cytokines, cytokine gene polymorphisms, and symptoms of potential acute coronary syndrome (ACS). Participants included 438 patients presenting to the emergency department (ED) whose symptoms triggered a cardiac evaluation (206 ruled in and 232 ruled out for ACS). Presence or absence of 13 symptoms was recorded upon arrival. Levels of tumor necrosis factor α (TNF-α), interleukin (IL)-6, and IL-18 were measured for all patients. A pilot analysis of 85 patients (ACS = 49; non-ACS = 36) genotyped eight single-nucleotide polymorphisms (SNPs; four *TNF* and four *IL6* SNPs). Logistic regression models were tested to determine whether cytokines or SNPs predicted symptoms. Increased levels of TNF-α and IL-6 were associated with a decreased likelihood of chest discomfort for all patients. Increased levels of IL-6 were associated with a lower likelihood of chest discomfort and chest pressure for ACS patients, and an increased likelihood of shoulder and upper back pain for non-ACS patients. Elevated IL-18 was associated with an increased likelihood of sweating in patients with ACS. Of the four *TNF* SNPs, three were associated with shortness of breath, lightheadedness, unusual fatigue, and arm pain. In all, protein cytokines and *TNF* polymorphisms were associated with 11 of 13 symptoms assessed. Future studies are needed to determine the predictive ability of cytokines and related SNPs for a diagnosis of ACS or to determine whether biomarkers can identify patients with specific symptom clusters.

**Keywords**

symptoms, acute coronary syndrome, single-nucleotide polymorphisms, cytokines, inflammation

Inflammatory processes play a central role in the pathogenesis and development of coronary atherosclerotic diseases (CAD) such as acute coronary syndrome (ACS). Research in a variety of clinical settings has demonstrated that increased levels of inflammatory markers are associated with increased future cardiovascular risk (Ait-Oufella, Taleb, Mallat, & Tedgui, 2011; Blake & Ridker, 2001).

Cytokines and their receptors are classes of polypeptides that mediate inflammatory processes (Verri et al., 2006). Pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, and IL-18 promote systemic inflammation. Anti-inflammatory cytokines suppress the activity of pro-inflammatory cytokines (Seruga, Zhang, Bernstein, & Tannock, 2008; Verri et al., 2006). While vast amounts of data support the link between inflammatory cytokines and atherosclerosis, no studies have demonstrated an association with symptoms of ACS. Authors have hypothesized that increased levels of cytokines influence and contribute to the sensation of pain by increasing the sensitization of nociceptors (Sturmer et al., 2005). Several studies have identified associations of TNF-α, IL-6, and IL-18 with atherogenesis and inflammation in cardiovascular disease (CVD), but many did not include symptoms as a potential confounding variable in statistical analyses (Blankenberg et al., 2006; Hansson, 2005; Mazzone et al., 2001; Schnabel & Blankenberg, 2009; Welsh et al., 2008). Researchers have examined the relationship between inflammatory markers and symptoms in patients with

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other chronic diseases and have had mixed results. Higher levels of TNF-α (Dowlati et al., 2010; Sukoff Rizzo et al., 2012) and IL-6 (Taraz, Taraz, & Dashti-Khavidaki, 2015) were associated with symptom burden in patients with a diagnosis of depression. In contrast, Miller, Freedland, and Carney (2005) found no association between the levels of in vitro cytokine biomarker production and depressive symptoms. X. S. Wang et al. (2010) found that patients with nonsmall cell lung cancer who were experiencing significant symptom burden, such as pain, fatigue, disturbed sleep, lack of appetite, and sore throat, during concurrent chemo and radiation therapy had significantly higher levels of IL-6 following treatment compared to before treatment. These results suggest a role for overexpressed pro-inflammatory cytokines in the worsening of physical symptoms.

Research has also established correlations between levels of the cytokine IL-18 and symptom profiles. IL-18 is capable of inducing both TNF-α and IL-6 expression (Mallat et al., 2002). In addition, authors have suggested that IL-18 plays a role in pain response in mice (Verri et al., 2008). Miyoshi, Obata, Kondo, Okamura, and Noguchi (2008) demonstrated that nerve injury in the spinal cord induced an increase in IL-18 and IL-18 receptor (IL-18R) expression.

Recent genome-wide association studies and meta-analyses have identified cytokine genes (*IL18*, *IL6*, and *TNF*) and their genetic variants as potential CAD risk loci, some of which significantly affect cytokine levels in serum (Cui et al., 2014; He et al., 2010; Tekola Ayele et al., 2012). Studies have confirmed that cytokine gene polymorphisms, located within critical promoter or regulatory regions of genes coding for cytokines, may have a significant influence on gene function. In addition, cytokine gene polymorphisms can affect gene transcription, resulting in changes in the expression of cytokines (Albert, 2011; Ansari, Humphries, Naveed, Khan, & Khan, 2017), and may represent genetic modifiers for a variety of common diseases including CAD (Johnson, Yucesoy, & Luster, 2004).

In addition to their role in the development of CAD, several gene polymorphisms, including variants in *TNF* (rs1800629, rs1799724, rs1799964, and rs361525) and IL-6 (rs1800795, rs1800796, rs1800797, and rs2069832), have been linked to both neuropathic and inflammatory pain symptoms (Belfer et al., 2004). These findings suggest that variations in both pro- and anti-inflammatory cytokine genes and protein cytokines may influence the symptom experience. Studies in patients with cancer have demonstrated relationships among variations in cytokines and symptom generation, perception, and expression (Doong et al., 2015; Reyes-Gibby et al., 2008). Dunn et al. (2013) found that polymorphisms in the IL-1 receptor 2 gene (*IL1R2*), *IL10*, and *TNF* were associated with depressive symptoms. Additionally, studies have found that variations in pro- and anti-inflammatory cytokine genes are associated with a symptom cluster of pain, fatigue, sleep disturbance, and depression in cancer patients (Doong et al., 2015; Reyes-Gibby et al., 2013).

Inflammatory cytokines have emerged as major contributors to the inflammatory process, and most published studies have shown a correlation between cytokines and CAD development (Castillo et al., 2010; Gori et al., 2005). Genetic polymorphisms and phenotypic markers also contribute to ACS severity and may therefore affect pain and associated symptoms (Berg et al., 2009). However, studies have yet to establish relationships between cytokine levels and cytokine genotypes and pain or other symptoms of ACS. Identification of biomarkers associated with pain and related symptoms of myocardial ischemia may help to identify individuals at high risk of certain symptoms. This knowledge could facilitate the development of more effective analgesics, alternative or complimentary therapies, and patient education. Therefore, the purpose of the present study was to determine whether relationships exist among protein cytokine levels (TNF-α, IL-6, and IL-18), cytokine gene polymorphisms, and symptoms of potential ACS.

## Method

### *Sample and Setting*

We enrolled patients who presented to the emergency department (ED) with symptoms triggering a cardiac evaluation. Enrollments occurred between January 2011 and December 2014 in five EDs in the Midwest, West, and Pacific Northwest regions of the United States. Patients were eligible to participate whether they were at least 21 years of age and fluent in English. We excluded patients if they experienced an exacerbation of heart failure (brain natriuretic peptide > 500 pg/ml), were transferred from a hemodialysis center, were referred for evaluation of a dysrhythmia, or had cognitive impairment, defined as the inability to understand and provide written informed consent. We also did not include patients if they had any immunologic dysfunction.

Of the 1,064 enrolled participants, we analyzed plasma cytokines for 438 and single-nucleotide polymorphisms (SNPs) for 85. The addition of the 85 patients for genetic analyses was made possible by a supplemental award from National Institute of Nursing Research. During patient interviews in the ED, we drew venous blood samples from all participants into a blue-top vacutainer tube (4.5 ml) for measurement of the cytokines TNF-α, IL-6, and IL-18. For the 85 participants for whom we were analyzing *TNF* and *IL6* polymorphisms, we drew an additional 4.5 ml into a yellow-top tube. We immediately placed the tubes on ice. In the processing laboratory at each site, blood samples for cytokine measurement were centrifuged at 3,000 rpm for 20 min to obtain platelet-poor plasma. Plasma was removed using a pipette and transferred to Eppendorf tubes in aliqouts of 500 µl. Specimens were capped; labeled with patient's name, study number, and date and time of blood draw; and stored in a laboratory freezer at −80 °C. The frozen samples were shipped to the study lab on dry ice for batch analyses. For the cytokine gene polymorphisms, we analyzed the whole blood using a procedure described below.

## Instruments

*ACS symptom checklist.* We measured symptoms dichotomously (yes/no) with the 13-item ACS Symptom Checklist (DeVon, Ryan, Ochs, & Shapiro, 2008). The checklist assesses for the presence or absence of 13 symptoms: chest pressure, shoulder pain, sweating, palpitations, chest discomfort, upper back pain, short of breath, arm pain, unusual fatigue, nausea, light headed, chest pain, and indigestion. Symptoms not included on the checklist are recorded in a blank space marked "other." The checklist has demonstrated reliability (Cronbach's $\alpha = .81$; DeVon, Ryan, Rankin, & Cooper, 2010) and validity (content validity indexes of .88 and .94), and investigators used it in previous studies in patient populations (DeVon et al., 2010; DeVon & Zerwic, 2003). The instrument is designed for the analysis of each symptom individually; there is no summary score.

*ACS Patient Information Questionnaire.* The ACS Patient Information Questionnaire includes patient-reported information on demographic, clinical, and symptom variables including overall symptom distress. Overall symptom distress is measured on scale of 1–10, with 1 representing lowest distress and 10 representing the worst overall symptom distress. The questionnaire was created using the standardized reporting guidelines recommended for studies evaluating risk stratification of ED patients with potential ACS (Hollander et al., 2004). It is designed to be self-administered, but for this study, the research specialist interviewed and recorded the patients' response to each item. The guidelines were established by the Multidisciplinary Standardized Reporting Criteria Task Force and are supported by the Society for Academic Medicine, American College of Emergency Physicians, American Heart Association, and American College of Cardiology. The goal of the questionnaire was to establish standardized ED reporting criteria that will facilitate study comparisons and meta-analyses.

*Charlson Comorbidity Index (CCI).* We measured comorbid conditions with the CCI), an instrument that predicts 1-year mortality risk based on burden of disease (Charlson, Szatrowski, Peterson, & Gold, 1994; Quan et al., 2011). The CCI provides a composite score calculated as a weighted sum of 19 comorbid conditions. Scores range from 0 to 35. A higher score can indicate a higher number of comorbid conditions or more severe comorbid conditions (e.g., *mild renal disease* = 1 and *metastatic cancer* = 6). Generally, higher scores represent a greater burden of disease. The instrument allows for the calculation of 1-year mortality based on illness severity (mild, moderate, and severe), reason for admission, and the weighted comorbidity score. It has been used extensively to quantify risk associated with comorbid conditions (De Groot, Beckerman, Lankhorst, & Bouter, 2003; Goldstein, Samsa, Matchar, & Horner, 2004) and is a reliable and valid prognostic indicator for in-hospital and 1-year outcomes in ACS patients (Nunez et al., 2004; Radovanovic et al., 2014).

*Duke Activity Status Index (DASI).* We measured functional status with the 12-item DASI. The DASI measures perceived functional capacity of patients with CVD based on the patient's ability to perform activities of daily living (Hlatky et al., 1989). Each response on the DASI, scored from 1 to 4, is weighted based on the known metabolic cost of each activity (Hlatky et al., 1989). Composite scores range from 0 to 58.2, with higher scores representing better physical functioning. Items reflect metabolic energy expenditure and correlate highly with peak $VO_2$ ($r = .80$, $p < .0001$; Hlatky et al., 1989) in patients with ACS (Katz et al., 2008).

## Procedures

The institutional review boards at the sponsoring institution and each data collection site approved this study and granted a waiver of initial consent for research staff to complete the ACS Symptom Checklist shortly after the patient was evaluated in triage. Research staff were blinded to each patient's final diagnosis. We enrolled patients between 7 a.m. and 11 p.m. every day of the week and assessed symptoms within 15 min of ED presentation in most cases. Research staff approached the patient for enrollment after they were admitted to a private examination room in the ED. We explained the purpose of the study, obtained written informed consent, and then recorded additional clinical and individual characteristics on the ACS Patient Information Questionnaire.

*Cytokine protein and SNP selection.* We chose to evaluate TNF-α, IL-6, and IL-18 plasma cytokine levels because these cytokines are elevated in patients with ACS and have been linked with atherogenesis and inflammation in CVD (Buraczynska, Ksiazek, Zukowski, & Grzebalska, 2016; Hussain, Iqbal, & Javed, 2015). We selected four *TNF* and four *IL6* SNPs (Table 1) for analysis based on their reported associations with inflammatory processes and CVD (Babu et al., 2012; Lio et al., 2004; Rehman et al., 2013). These variant SNPs are common in the American population, having at least 5% minor allele frequency (MAF) and representation across racial groups. Throughout this article, we will refer to the allele that is less common in the population as the minor allele and to the more common, or "wild-type" allele, as the major allele. Aside from one intronic SNP (*IL6* rs2069832), all other SNPs were intergenic and located at the 5-prime region of the *TNF* or *IL6* gene, as annotated in Stanford's University of California Santa Cruz (UCSC) Genome Browser. While these SNPs have no obvious functional impact on the resulting amino acid chain or final protein, their presence in the 5-prime region proximal to the gene(s) may be important, as these regions are known for their involvement in gene signaling.

*Cytokine and DNA processing.* Plasma samples were analyzed for TNF-α, IL-6, and IL-18 cytokine levels using enzyme-linked immunosorbent assay kits manufactured by R&D Systems (Minneapolis, MN). The precision and sensitivity of each kit were as follows: (1) For TNF-α, intra-assay precision was 4–6%,

**Table 1.** Dominant Model Genotype Frequencies Among Participants ($N = 85$) for Cytokine Single-Nucleotide Polymorphisms (SNPs).

| SNP (Minor Allele) and Genotype | *n* (%) |
|---|---|
| *TNF* | |
| rs1800629 (G) | |
| AA | 37 (43.5) |
| AG[a] | 48 (56.5) |
| rs1799724 (T) | |
| CC | 68 (80.0) |
| CT[a] | 17 (20.0) |
| rs1799964 (C) | |
| TT | 57 (67.1) |
| CT/CC | 28 (32.1) |
| rs361525 (A) | |
| GG | 74 (89.2) |
| GA[a] | 9 (10.8) |
| *IL6* | |
| rs1800795 (C) | |
| GG | 38 (45.2) |
| GC/CC | 46 (54.8) |
| rs1800796 (C) | |
| GG | 62 (73.8) |
| GC/CC | 22 (26.2) |
| rs1800797 (G) | |
| AA | 45 (52.9) |
| AG[a] | 40 (47.1) |
| rs2069832 (A) | |
| GG | 37 (44.0) |
| AG/AA | 47 (55.9) |

[a]Indicates no homozygous minor allele carriers in sample.

interassay precision was 4–8%, the minimum detectable level was 1.5 pg/ml, and the maximum detectable level was 5,000 pg/ml. (2) For IL-6, intra-assay precision was 2–6%, interassay precision was 2–8%, the minimum detectable level was 1.0 pg/ml, and the maximum detectable level was 2,000 pg/ml. (3) For IL-18, intra-assay precision was 5–10%, interassay precision was 5–10%, the minimum detectable level was 12.5 pg/ml, and the maximum detectable level was 5,000 pg/ml.

DNA was isolated from whole-blood samples using the Puregene (Gentra Biosystems) DNA isolation kit. The standard protocol involves cell lysis, proteinase K treatment, protein precipitation, and DNA precipitation. DNA was resuspended in Tris-EDTA buffer for long-term storage. Isolated DNA was stored in the freezer at −20°C. Genotyping was performed using Sequenom MassARRAY™. iPLEX™ assays were designed utilizing the Sequenom Assay Design software, version 3.1, allowing for single-base extension (SBE) designs used for multiplexing. Multiplex assays were performed to amplify 5–10 ng of genomic DNA by polymerase chain reaction (PCR). PCR were treated with shrimp alkaline phosphatase to neutralize unincorporated deoxynucleotide triphosphates (dNTPs). Subsequently, a post-PCR SBE reaction was performed for each multiplex reaction using concentrations of .625 μM for low-mass primers and 1.25 μM for high-mass primers. Reactions were diluted with 16 μl of $H_2O$, fragments were purified with resin, spotted onto Sequenom SpectroCHIP™ microarrays, and scanned by Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry. Individual SNP genotype calls were then generated using Sequenom TYPER™ software, version 4.0, which automatically calls allele-specific peaks according to their expected masses. A lab technician evaluated and checked each set of calls per run for call quality. Quality control checks were in place at every step: Multiple samples (10%) were routinely genotyped in duplicate in each plate of DNA. Cases and unaffected controls were gridded together in each plate to avoid any systematic biases between plates. We removed individuals with low genotype call rates (<95%) and SNPs with low call rates (<90%).

## Statistical Analyses

Data analyses were performed using SPSS Version 22.0 (IBM Corp, Armonk, NY) and SAS Version 9.4 (SAS®, Cary, NC). Significance was set at $p < .05$ for all statistical procedures. Statistical procedures were uncorrected for multiple testing as the analyses were exploratory in nature. Descriptive statistics and frequency distributions were generated to assess sample characteristics.

*Protein cytokine analyses.* Logistic regression tests were run to determine whether cytokine levels predicted risk of symptoms. Each symptom was included as the dependent outcome for two models: (1) a model with all patients combined and (2) a model with an ACS diagnosis-by-cytokine interaction. Odds ratios (*OR*s) and 95% confidence intervals (CIs) were calculated from these models for the full sample and by ACS diagnosis. *OR*s for TNF-α represent the change in the odds of a symptom being present for a 10-unit increase in cytokine level. *OR*s for IL-6 and IL-18 represent the change in the odds of a symptom being present for a 100-unit increase in cytokine level. Variables known to affect symptom presentation and/or inflammation among ACS patients were selected as covariates for the models and included age, sex, race, scores on the CCI and the DASI, and time of day of ED admission. Models were adjusted for time of admission because cytokines exhibit a diurnal pattern of expression (Altara et al., 2015; Galbo & Kall, 2016; Nilsonne, Lekander, Åkerstedt, Axelsson, & Ingre, 2016). Time of day was collapsed into four 6-hr categories to encompass peaks and troughs in cytokine levels occurring in the early morning and in the afternoon or evening. Blood for analysis was drawn at time of ED admission because our intent was to determine whether there was an association between selected cytokines and acute symptoms.

*Genetic analyses.* Hardy-Weinberg equilibrium (HWE) tests were performed for each SNP to assess for deviations from expected population frequencies. *TNF* SNP rs1800629 and *IL6* SNP rs1800797 demonstrated significant deviations from HWE ($p < .0001$ and $p < .005$, respectively). For these two variants, the major and minor alleles were opposite of what the 1000 Genomes Project reported (1000 Genomes Project

Consortium, 2015). This difference was not due to reverse-strand genotyping, nor did it appear to be due to population stratification differences. Due to these unexplained discrepancies, we excluded *TNF* rs1800629 and *IL6* rs1800797 from our genetic analyses. Among all other SNPs, racially stratified MAFs were consistent with those reported by 1000 Genomes (1000 Genomes Project Consortium, 2015). Due to small sample sizes for minor allele carrier status, dominant genetic analyses were performed for all SNPs. In these analyses, heterozygous genotypes (Aa) and homozygous minor genotypes (aa) were collapsed into a single category then compared to the reference homozygous major genotype (AA) carriers. Analyses were not stratified or adjusted for racial or ethnic characteristics due to small sample size and because the minor allele for the SNPs being assessed did not vary by racial group.

To test the prediction of ACS symptoms by SNP minor allele presence, we performed logistic regression analyses that were similar to the cytokine plasma models. Each symptom was modeled as a dependent outcome for (1) a model with the full sample of patients and (2) a model with the with following independent variables: SNP (dominant), ACS case status, and an SNP-by-ACS (multiplicative) interaction term. *OR*s and 95% CIs were calculated from these models for the full sample and by ACS diagnosis. *OR*s represent the change in odds of reporting a symptom when the minor allele for an SNP is present. Analyses were unadjusted for covariates due to small sample size.

## Results

### Sample Demographics

Demographic and clinical characteristics of the 438 participants included in the cytokine plasma analysis and the 85 participants included in the SNP analysis appear in Table 2. We analyzed both cytokine and SNP samples in 49 of the participants. The sample demographics were consistent with regional and national characteristics of ACS and non-ACS cohorts. Most participants had private insurance or Medicare, greater than a higher school education (at least some college or higher), and an income of $\geq$US$20,000. The majority of patients had hypertension, and slightly less than half had previously used tobacco or were obese. ACS cases were more likely than non-ACS cases to be male and have hypertension and hypercholesterolemia, and a majority were diagnosed with non-ST elevation myocardial infarction.

### Protein Cytokines and Symptoms

Results for the effects of cytokines on ACS symptoms appear in Table 3. For statistical models including all patients, both with and without ACS, higher TNF-$\alpha$ levels and higher IL-6 levels were associated with lower odds of experiencing chest discomfort. Higher IL-6 levels were associated with increased odds of upper back pain, and higher IL-18 levels were associated with increased odds of nausea.

To further explore the effects of ACS diagnosis on the cytokine–symptom relationship, we tested interaction models with cytokine-by-ACS interaction terms and evaluated the diagnosis specific and interaction effects on reported symptom. For IL-6 levels, the odds of chest discomfort and chest pressure were significantly lower with higher cytokine levels among ACS cases, but not among controls, with greater evidence of an interaction effect of IL-6-by-ACS status in the association of chest pressure ($p_{\text{interaction}} = .008$). Similarly, for IL-18 levels, the significantly increased likelihood of sweating with higher cytokine levels was uniquely observed among ACS cases but with a nonsignificant interaction term. The finding of increased sweating with elevated IL-18 is noteworthy since sweating is associated with myocardial infarction (Ryan et al., 2007). In some cases, models were only significant among patients for whom ACS was ruled out. Increased odds of shoulder pain and upper back pain were associated with elevated IL-6 levels in patients in which ACS was ruled out. Higher IL-18 levels were associated with decreased odds of palpitations in patients without ACS.

### SNPs and Symptoms

Results for analyses of the effects of SNPs on ACS symptoms appear in Table 4. These pilot results should be viewed with caution due to the small sample sizes and observation of wide 95% CIs. For models combining both ACS and non-ACS patients, the presence of any copy of the minor allele for *TNF* rs1799964 (C) and *TNF* rs361525 (A) was associated with increased odds of reporting light headedness. Analyses with SNP-by-ACS diagnosis interaction terms revealed how effects differed by ACS diagnosis. Notably, the presence of any copy of the *TNF* rs1799724 minor allele (T) was associated with greater odds of shortness of breath in patients diagnosed with ACS. The *TNF* rs1799964 minor allele (C) was significantly associated with higher rates of unusual fatigue among ACS cases but not non-ACS controls, with consistent directions of effect among the groups. *TNF* rs361525 minor allele (A) was associated with greater odds of reporting arm pain for patients without ACS. There were no IL-6 SNPs associated with ACS symptom presentation.

## Discussion

The purpose of the present study was to determine whether relationships existed between protein cytokine levels (TNF-$\alpha$, IL-6, and IL-18) or cytokine gene polymorphisms and symptoms of potential ACS. Key cytokine findings were that increased levels of TNF-$\alpha$ and IL-6 were associated with a decreased likelihood of chest discomfort for all patients, an increased level of IL-6 was associated with a lower likelihood of chest pressure for ACS patients only, elevated IL-18 level was associated with an increased likelihood of sweating in patients with ACS only. We explored genotype associations with self-reported symptoms for six of eight common candidate SNP variants meeting the HWE assumption. Of these, three

**Table 2.** Sample Characteristics.

| Characteristic | ACS | | Non-ACS | |
|---|---|---|---|---|
| | Cytokine Sample ($n = 206$) | SNP Sample ($n = 49$) | Cytokine Sample ($n = 232$) | SNP Sample ($n = 36$) |
| Age (years), mean (*SD*) | 60.7 (11.4) | 63 (10.1) | 58.2 (15.5) | 58.7 (16.5) |
| Sex (male), *n* (%) | 154 (74.8) | 30 (61.2) | 120 (51.7) | 23 (63.9) |
| Diagnosis, *n* (%) | | | | |
| UA | 46 (22.3) | 8 (16.3) | na | na |
| NSTEMI | 123 (59.7) | 34 (69.4) | na | na |
| STEMI | 37 (18.0) | 7 (14.3) | na | na |
| Race, *n* (%) | | | | |
| White | 134 (65.0) | 30 (61.2) | 164 (71.3) | 27 (75.0) |
| Black | 34 (16.5) | 1 (2.0) | 23 (10.0) | 2 (5.6) |
| Hispanic | 15 (7.3) | 15 (30.6) | 19 (8.3) | 6 (16.7) |
| Other | 23 (11.2) | 3 (6.1) | 24 (10.4) | 1 (2.8) |
| Health insurance, *n* (%) | | | | |
| Private from employer | 75 (36.9) | 14 (29.2) | 74 (32.5) | 13 (36.1) |
| Private paid by patient | 10 (4.9) | 4 (8.3) | 21 (9.2) | 4 (11.1) |
| Medicare | 61 (30.0) | 20 (41.7) | 81 (35.5) | 14 (38.9) |
| Other government insurance[a] | 22 (10.8) | 5 (10.4) | 28 (12.3) | 4 (11.1) |
| Not insured | 35 (17.2) | 5 (10.4) | 24 (10.5) | 1 (2.8) |
| Education, *n* (%) | | | | |
| <High school | 27 (13.2) | 13 (27.1) | 25 (10.8) | 4 (11.1) |
| High school | 46 (22.4) | 8 (16.7) | 44 (19.0) | 7 (19.4) |
| Some college | 61 (29.8) | 10 (20.8) | 77 (33.3) | 8 (22.2) |
| College/graduate work | 44 (21.5) | 12 (25.0) | 47 (20.3) | 7 (19.4) |
| Graduate degree | 27 (13.2) | 5 (10.4) | 38 (16.4) | 10 (27.8) |
| Income, *n* (%) | | | | |
| <20,000 | 52 (27.1) | 12 (30.0) | 73 (33.8) | 6 (17.6) |
| 20,000–49,999 | 64 (33.3) | 11 (27.5) | 80 (37.0) | 17 (50.0) |
| 50,000–99,999 | 43 (22.4) | 10 (25.0) | 33 (15.3) | 5 (14.7) |
| ≥ 100,000 | 33 (17.2) | 7 (17.5) | 30 (13.9) | 6 (17.6) |
| Hypertension, *n* (%) | 144 (70.2) | 31 (64.6) | 139 (60.4) | 21 (58.3) |
| Hypercholesterolemia, *n* (%) | 128 (64.0) | 30 (65.2) | 113 (50.9) | 21 (58.3) |
| Family history,[b] *n* (%) | 88 (44.9) | 23 (48.9) | 112 (49.3) | 18 (51.4) |
| Diabetes, *n* (%) | 57 (27.7) | 14 (28.6) | 67 (29.0) | 11 (30.6) |
| Tobacco use, *n* (%) | 103 (50.5) | 25 (52.1) | 97 (42.2) | 15 (42.9) |
| Cocaine use, *n* (%) | 19 (9.3) | 1 (2.0) | 15 (6.5) | 0 (0.0) |
| Kidney disease, *n* (%) | 22 (10.8) | 5 (10.2) | 26 (11.3) | 8 (22.2) |
| Obese, *n* (%) | 90 (43.7) | 22 (45.8) | 103 (44.4) | 20 (55.6) |
| DASI, mean (*SD*) | 37.6 (18.3) | 41 (18.6) | 32.1 (19.4) | 34.9 (20.8) |
| CCI, mean (*SD*) | 2 (1.7) | 1.7 (1.2) | 1.7 (2.0) | 1.3 (1.6) |
| Symptoms, *n* (%) | | | | |
| Chest discomfort | 138 (67.0) | 34 (69.4) | 163 (70.3) | 23 (63.9) |
| Chest pain | 144 (69.9) | 31 (63.3) | 155 (66.8) | 23 (63.9) |
| Chest pressure | 142 (68.9) | 33 (67.3) | 153 (65.9) | 20 (55.6) |
| Short of breath | 103 (50.0) | 30 (61.2) | 141 (60.8) | 17 (47.2) |
| Unusual fatigue | 77 (37.4) | 26 (53.1) | 122 (52.6) | 16 (44.4) |
| Lightheadedness | 72 (34.9) | 21 (43.7) | 104 (44.8) | 16 (44.4) |
| Nausea | 66 (32.0) | 25 (51.0) | 89 (38.4) | 13 (36.1) |
| Arm pain | 73 (35.4) | 28 (57.1) | 69 (29.7) | 8 (22.2) |
| Sweating | 67 (32.5) | 24 (49.0) | 67 (28.9) | 12 (33.3) |
| Shoulder pain | 66 (32.0) | 24 (49.0) | 69 (29.7) | 9 (25.0) |
| Upper back pain | 40 (19.4) | 19 (38.8) | 72 (31.0) | 9 (25.0) |
| Palpitations | 53 (25.7) | 10 (20.4) | 70 (30.2) | 14 (38.9) |
| Indigestion | 41 (19.9) | 23 (46.9) | 53 (22.8) | 13 (36.1) |
| Symptom number, mean (*SD*) | 5.2 (3.1) | 6.7 (3.3) | 5.7 (3.1) | 5.4 (3.0) |
| Overall symptom distress, mean (*SD*) | 7.1 (2.6) | 7.5 (2.5) | 6.7 (2.5) | 6.4 (2.6) |
| Cytokine level, mean (*SD*) | | | | |
| TNF-α (pg/ml) | 3 (5.9) | 2.2 (1.3)[c] | 3.3 (5.5) | 2.7 (2.6)[d] |
| IL-6 (pg/ml) | 25.9 (62.6) | 18.3 (31.3)[c] | 36.2 (90.4) | 24.6 (52.2)[d] |
| IL-18 (pg/ml) | 218 (162.5) | 257.8 (184.2)[c] | 234.1 (224.6) | 200.5 (101.0)[d] |

*Note.* Cytokine sample includes participants that had blood collected for cytokine testing. The SNP sample includes participants that had blood collected for genetic testing. ACS = acute coronary syndrome; CCI = Charlson Comorbidity Index; DASI = Duke Activity Status Index; NA = not applicable; NSTEMI = non-ST elevation myocardial infarction; SNP = single-nucleotide polymorphism; STEMI = ST elevation myocardial infarction; UA = unstable angina; IL = interleukin.
[a]Veteran's Administration (VA)/disability/Medicaid values represent *n* (%) unless otherwise denoted. [b]Family history of heart disease or premature sudden cardiac death. [c]*n* is 25. [d]*n* is 24.

**Table 3.** Prediction of Symptoms From Cytokine Levels.

| | | Participants | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | All | | | ACS Cases ($n = 206$) | | | Non-ACS Controls ($n = 232$) | | | Interaction |
| Cytokine | Symptom | *n* | Odds Ratio (95% CI) | *p* Value | *n* | Odds Ratio (95% CI) | *p* Value | *n* | Odds Ratio (95% CI) | *p* Value | *p* Value |
| TNF-α | Chest discomfort | 433 | 0.6 [0.4, 0.9] | **.017** | 206 | 0.5 [0.2, 1.2] | .109 | 227 | 0.6 [0.3, 1.1] | .077 | .612 |
| | Shoulder pain | 433 | 1.3 [0.9, 1.8] | .143 | 206 | 0.8 [0.4, 1.6] | .533 | 227 | 2.4 [1.2, 4.7] | .010 | **.025** |
| IL-6 | Chest discomfort | 432 | 0.7 [0.5, 0.9] | **.019** | 206 | 0.4 [0.1, 0.8] | **.016** | 226 | 0.8 [0.6, 1.1] | .184 | .070 |
| | Chest pressure | 432 | 0.8 [0.6, 1.0] | .097 | 206 | 0.3 [0.1, 0.7] | **.005** | 226 | 1.0 [0.7, 1.4] | .895 | **.008** |
| | Shoulder pain | 432 | 1.1 [0.9, 1.4] | .342 | 206 | 0.6 [0.3, 1.3] | .195 | 226 | 1.4 [1.0, 1.9] | **.046** | .054 |
| | Upper back pain | 432 | 1.3 [1.0, 1.7] | **.031** | 206 | 1.1 [0.6, 1.9] | .721 | 226 | 1.4 [1.0, 2.0] | **.040** | .436 |
| IL-18 | Nausea | 433 | 1.1 [1.0, 1.3] | **.045** | 206 | 1.1 [0.9, 1.3] | .268 | 227 | 1.1 [1.0, 1.3] | .095 | .886 |
| | Sweating | 433 | 1.1 [1.0, 1.2] | .086 | 206 | 1.3 [1.1, 1.5] | **.010** | 227 | 1.0 [0.9, 1.2] | .638 | .058 |
| | Palpitations | 433 | 0.9 [0.8, 1.0] | .051 | 206 | 1.0 [0.8, 1.2] | .804 | 227 | 0.8 [0.6, 1.0] | **.025** | .151 |

*Note.* Table presents two models for each symptom outcome: (1) a model with all patients combined and (2) a model with an ACS diagnosis-by-cytokine interaction and separate odds ratios (*OR*s) calculated for ACS and non-ACS patients. Models adjusted for age, sex, race, Charlson Comorbidity Index, Duke Activity Status Index, and time of day. *OR* for TNF-α represents change in odds of symptom being present for 10-unit increase in cytokine level. *OR* for IL-6 and IL-18 represents change in odds of symptom being present for 100-unit increase in cytokine level. All 13 symptoms from ACS Symptoms Checklist were tested. Only symptoms with statistically significant comparisons are shown. Boldface values indicate significance at $\alpha < .05$. ACS = acute coronary syndrome; CI = confidence interval; IL = interleukin; TNF-α = tumor necrosis factor-α.

*TNF* SNPs were associated with shortness of breath, light headedness, unusual fatigue, and arm pain.

Evidence from prior studies suggests that changes in TNF-α protein levels are associated with symptoms such as pain, fatigue, sleep disturbance, and depression. In one study, expression of TNF-α increased in neurons following a painful stimulus (Andrade et al., 2011). Koch et al. (2007), studying 94 patients with chronic pain, found that levels of pro-inflammatory cytokines, including TNF-α and IL-6 in the plasma, correlated with elevated pain intensity. Their study included a heterogeneous sample of patients with a variety of chronic pain disorders, such as postherpetic neuralgia, complex regional pain syndrome, cancer pain, arthrosis, back pain, failed back surgery syndrome, and fibromyalgia. H. Wang, Schiltenwolf, and Buchner (2008), in a prospective longitudinal clinical study, evaluated the role and clinical relevance of TNF-α in patients with chronic low-back pain. They found that a significantly higher proportion of patients with chronic low-back pain had increased TNF-α levels during a 6-month course compared to a healthy control group. Unlike in previous studies, Imholz et al. (2017) found no significant association between chest pain and either TNF-α or IL-6 levels in patients with myocardial infarction. They suggested that during the period between symptom onset and blood collection, pro-inflammatory cytokines may have been downregulated by anti-inflammatory mediators, making any initial association between levels of these cytokines and pain intensity difficult to detect. By contrast, in participants in the present study, higher levels of TNF-α and IL-6 were associated with lower odds of experiencing chest discomfort in patients with and without ACS. It is possible that this finding could be explained by the presence of anti-inflammatory cytokines, which we did not measure. When tissue is invaded or destroyed by leukocytes during an inflammatory episode, several mediators such as IL-1, IL-6, and TNF-α-1 migrate to the site (Rittner, Machelska, & Stein, 2005), contributing to pain sensations. At the same time, some analgesic mediators are also released, which may mitigate the stimulation of pain sensors (DeVon, Piano, Rosenfeld, & Hoppensteadt, 2014).

Weber et al. (2016) examined levels of IL-6 in patients with disc herniation, spinal stenosis, or degenerative disc disease compared to levels in healthy controls. They found that levels of IL-6 were significantly higher in participants with lower back pain. Individuals with lower back pain from spinal stenosis or degenerative disc disease had significantly higher levels of IL-6 than both those with pain from disc herniation and healthy controls. In the present study, we observed similar increased reports of upper back pain (rather than lower) with increased levels of IL-6 in the non-ACS group. Higher levels of IL-6 in patients ruled in for ACS, however, were associated with lower odds of experiencing chest discomfort or chest pressure. Despite this reduced likelihood of chest-related symptoms, ACS case status contributed uniquely to these models; significant interaction effects of case status and chest pressure further strengthens this evidence.

Elevated IL-18 contributes to both the central and peripheral stress responses, and researchers found that panic attacks in humans or restraint stress in mice induce a rapid increase in the level of circulating IL-18 (Kokai, Kashiwamura, Okamura, Ohara, & Morita, 2002). These authors suggested that the elevation of plasma IL-18 levels reflects the increased production and release of IL-18 in the central nervous system under stressful settings. In the present study, we found that, among the combined case/control sample, increased IL-18 levels were significantly associated with increased report of nausea, yet our post hoc findings were not informative regarding group effects. Only among patients with ACS were higher levels of IL-18

**Table 4.** Prediction of Symptoms by *TNF* Genotype (Dominant Genetic Models).

| *TNF* Genotype Definition | | | | | | | All | | | ACS Cases | | | Non-ACS Controls | | | Interaction |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP | Variant Location | Loci (GRCh38.p12) | Minor Allele | Major Allele | MAF | Symptom | *n* | Odds Ratio (95% CI) | *p* Value | *n* | Odds Ratio (95% CI) | *p* Value | *n* | Odds Ratio (95% CI) | *p* Value | *p* Value |
| rs1799724 | Intergenic: 5′ upstream *TNF* | 31574705 | T | C | .10 | Short of breath | 85 | 3.2 [1.0, 11.0] | .058 | 49 | 10.4 [1.2, 89.1] | **.032** | 36 | 0.7 [0.1, 4.9] | .728 | .068 |
| rs1799964 | Intergenic: 5′ upstream *TNF* | 31574531 | C | T | .17 | Lightheaded | 85 | 3.5 [1.4, 9.1] | **.010** | 49 | 3.3 [0.9, 12.1] | .072 | 36 | 3.9 [0.9, 15.9] | .061 | .226 |
| | | | | | | Unusual fatigue | 85 | 2.5 [1.0, 6.3] | .058 | 49 | 4.9 [1.2, 20.7] | **.031** | 36 | 1.4 [0.4, 5.6] | .593 | .061 |
| rs361525 | Intergenic: 5′ upstream *TNF* | 31575324 | A | G | .05 | Lightheaded | 83 | 5.6 [1.1, 29.0] | **.039** | 48 | 6.5 [0.7, 63.4] | .107 | 35 | 4.7 [0.4, 51.1] | .199 | .852 |
| | | | | | | Arm pain | 83 | 2.9 [0.7, 12.6] | .149 | 48 | 1.1 [0.2, 7.1] | .936 | 35 | 15.6 [1.3, 182.1] | **.028** | .091 |

*Note.* Table presents two models for each symptom outcome: (1) a model with all patients combined and (2) a model with an ACS diagnosis-by-SNP interaction and separate *OR*s calculated for ACS and non-ACS patients. *TNF* SNP rs1800629 was not analyzed due to violation of Hardy–Weinberg equilibrium. All 13 symptoms from the ACS Symptom Checklist were tested. Only symptoms with statistically significant comparisons are shown. Boldface values indicate significance at $\alpha < .05$. ACS = acute coronary syndrome; CI = confidence interval; MAF = minor allele frequency; SNP = single-nucleotide polymorphism.

associated with higher odds of experiencing sweating, which is consistent with the existing literature (Ryan et al., 2007). Sweating during ACS is triggered by stimulation of the sympathetic nervous system. Based on Kokai et al.'s findings, we hypothesize that increases in IL-18 may occur following the stress of ACS, contributing to the occurrence or severity of sweating. Sweating and palpitations are also associated with anxiety and stress, so it is possible that IL-18 is activated by both ischemia and the mental stress of acute illness.

We observed significant preliminary symptom—*TNF* genotype associations in patients evaluated for ACS that warrant further research. Taken as a whole, our pilot gene associations were in the expected direction; that is, we observed increased risk of symptoms in the presence of *TNF* risk alleles. A number of studies found that, among cytokine SNPs, *TNF* rs1800629 and *IL6* rs1800795 were significantly associated with CAD, and these SNPs were important risk factors for the development of ACS (Ansari et al., 2017; Babu et al., 2012; Kazemi et al., 2018).

In a systematic review, authors found associations between elevated fatigue and specific polymorphisms in *TNF* genes (T. Wang, Yin, Miller, & Xiao, 2017). Cytokine SNPs were significantly associated with all three subgroups of fatigue (chronic fatigue syndrome, cancer-related fatigue, and other disease-related fatigue). The authors further found that a *TNF* gene variation may be associated with differences in frequency and severity of fatigue. Another study showed that the TNF SNP rs1800629 was associated with morning fatigue in breast cancer patients (Dhruva et al., 2015). Other researchers found that the same SNP was independently associated with fatigue in breast cancer survivors (Bower et al., 2013). Although we did not evaluate the rs1800629 variant in our study, we did identify a significant association of *TNF* SNP rs1799964 with unusual fatigue in ACS patients but not in non-ACS controls. However, the CI for this finding was very wide; thus, this association requires future study with larger samples. We identified a number of other associations between *TNF* SNPs and symptoms, including positive associations of rs17999964 and rs361525 with greater odds of reporting lightheadedness and of rs1799724 with increased odds of reporting shortness of breath. These are the first reports of associations between *TNF* variants and these symptoms and should be considered preliminary.

We found no significant associations of *IL6* SNPs with any symptoms, despite prior evidence in the literature. T. Wang et al. (2017) demonstrated that *IL6* SNP rs1800795 was associated with cancer-related fatigue during and after treatment. Similarly, Bower et al. (2013) found that the same SNP was independently associated with fatigue in breast cancer survivors. Shi et al. (2015) found that the IL6 GG genotype for rs1800795 showed diverse associations with moderate/severe symptoms by ethnic group in patients with multiple myeloma 1 year after diagnosis: Non-Hispanic Whites with a GG genotype were less likely to report moderate/severe fatigue, while that genotype predicted moderate/severe pain in patients other than non-Hispanic Whites with multiple myeloma 1 year postdiagnosis. Rausch et al. (2010) reported that dyspnea was

associated with *IL6* rs2069835 in Caucasian lung cancer survivors. The differences between our findings and those of these prior studies may be related to chronic versus acute symptom mechanisms. Or, it may be that studies powered to assess differences by subgroups are needed to reveal significant associations between *IL6* SNPs and patient symptoms.

It is important to note that, in our review of the literature regarding relationships between pain and pain-associated symptoms and cytokines, a vast majority of studies enrolled patients with chronic pain. The causes of pain vary in chronic versus acute conditions. In ischemic cardiac pain, there is stimulation of the autonomic nervous system (DeVon et al., 2014). By contrast, the cause of pain in many chronic pain conditions is neuropathic. Therefore, associations between cytokines and pain may vary between acute and chronic conditions. The present study was of acute symptoms and acute pain phenotypes for ACS. We do not yet know whether biological pathways of pain are completely different or may overlap in acute or chronic painful conditions.

### *Limitations*

There were several limitations to the study. First, the genetic sample size was small. A larger sample may be needed to increase the power to detect differences in associations for other cytokine gene polymorphisms. Second, we did not adjust statistical analyses for the genetic results for covariates due to the small sample size of the SNP models. Third, we did not correct statistical procedures for multiple testing as the analyses were exploratory in nature. Fourth, the sample was predominantly Caucasian; therefore, generalizability of our findings to other racial or ethnic groups is limited. Five, we cannot infer cause-and-effect relationships among symptoms, genotype, and inflammatory protein levels through this observational study. We also acknowledge that patients presenting with symptoms suggestive of ACS but subsequently ruled out for the condition are not optimal controls. Some of these patients had chronic ischemic heart disease but did not experience ACS on that admission. A healthy control group may have strengthened the internal validity of the findings. Despite these limitations, we were able to detect significant associations between protein cytokines and SNPs and selected symptoms in patients evaluated for ACS.

## Conclusions

In the present study, we found that select cytokine plasma levels and cytokine gene polymorphisms were associated with 11 of the 13 ACS symptoms we assessed. Our goal was to seek preliminary evidence of associations between circulating and genetic biomarkers with symptoms of ACS that could be relevant for risk screening and improved diagnosis in the future. We focused on cytokine-related biomarkers due to their known involvement in CAD and their existing association with other (chronic) symptoms. Inflammatory biomarkers could be useful for discriminating patients with less typical ACS symptoms from those with other disease conditions, particularly among high-risk individuals. Our preliminary data may contribute to reducing the significant gap in our understanding of the influence of cytokine biomarkers on expression of acute symptoms. Our observed effect sizes, directions of effect, and SNP population estimates among ACS cases and controls can inform the design of future ACS–symptom–cytokine association studies.

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### Author Contributions

S. Mirzaei contributed to conception design, analysis, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. L. Burke contributed to design, analysis, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. A. Rosenfeld contributed to conception, design, and acquisition; drafted the manuscript; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. S. Dunn contributed to conception, design, acquisition, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. J. Dungan contributed to conception, design, analysis, and interpretation; drafted the manuscript; critically the revised manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. K. Maki contributed to conception acquisition, analysis, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. H. DeVon contributed to conception, design, acquisition, analysis, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy.

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