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RESEARCH ARTICLE

Screening the human serum proteome for genotype–phenotype associations: An analysis of the *IL6* –174G>C polymorphism

Christine M. Hegedus[1], Christine F. Skibola[1], Paige Bracci[2], Elizabeth A. Holly[2] and Martyn T. Smith[1]

[1] School of Public Health, University of California, Berkeley, CA, USA
[2] Department of Epidemiology and Biostatistics, University of California, San Francisco, CA, USA

Interleukin (IL)-6 is a circulatory, pleiotropic cytokine with multiple roles in the immune system. Both IL-6 and the *IL6* –174G>C promoter polymorphism have been linked to various diseases associated with inflammation. However, the mechanism by which the polymorphism influences disease risk is unclear. We postulated that serum proteome analysis of individuals with different *IL6* –174G>C genotypes would provide insight on genotype–phenotype associations of this polymorphism and its role in disease susceptibility. Serum from a random sample of control participants in an ongoing population-based case-control study of non-Hodgkin lymphoma was pooled by *IL6* genotype and used to screen for the optimal SELDI-TOF MS arrays for analysis. We report differences in serum protein expression of individuals with specific genotypes based on pooled and individual sample analysis. In particular, we report an association of the –174C allele with increased apolipoprotein C-I (ApoC-I). Additionally, we corroborate previous findings of an association of the –174C allele with lower autoantibodies to heat shock protein 60 and confirm the absence of any association between the *IL6* –174G>C genotype and serum IL-6 levels. This study illustrates that proteome analysis can enhance our understanding of genotype–phenotype relationships. Additional studies are needed to clarify the interaction between the *IL6* –174G>C polymorphism and ApoC-I.

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1 Introduction

Proteomics and genomics are rapidly growing fields that have proven useful for the identification and understanding of risk factors for various disease states. Surprisingly, there has been little integration of the two fields. Whereas functional studies have examined the effects of genetic polymorphisms on specific proteins and pathways, few studies have looked at the integrated effect of polymorphisms on expressed phenotypes. Analysis of the serum proteome can provide valuable information on polymorphism function as it contains peptides and proteins secreted by various tissues that may reflect an organism's pathophysiological state.

Interleukin (IL)-6 is a circulatory, pleiotropic cytokine with roles in innate and acquired immunity. Numerous cell types respond to IL-6 and a variety of responses can result, including activation of the acute phase response, cellular differentiation, and proliferation [1–5]. Recently, IL-6 has been targeted for its role in susceptibility to psoriasis and rheumatoid arthritis [6]. The presence of a G to C poly-

Correspondence: Christine M. Hegedus, School of Public Health, University of California, 140 Warren Hall, Berkeley, CA 94720, USA
E-mail: chegedus@berkeley.edu
Fax: +1-510-642-0427

Abbreviations: ApoC-I, apolipoprotein C-I; **HDL,** high-density lipoprotein; **HSP60,** heat shock protein 60; **IL,** interleukin; **LDL,** low-density lipoprotein; **MW,** molecular weight; **PF,** platelet factor

morphism in the *IL6* promoter at position –174 has been reported and the C allele has been inversely associated with systemic-onset juvenile chronic arthritis [7] and Kaposi sarcoma in HIV-positive men [8]. The C allele has also been associated with decreased levels of autoantibodies to human heat shock protein 60 (HSP60), which are thought to play a role in atherosclerosis [9, 10]. However, it is unknown if the association between the –174G>C polymorphism and disease risk is a direct or indirect result of IL-6 as there have been discrepancies about the effect of the polymorphism on circulating IL-6 levels [7, 9, 11, 12]. Thus, additional studies of the genotype–phenotype association of this polymorphism are necessary to gain insight into its role in disease susceptibility. We hypothesized that analysis of the serum proteome by *IL6* –174G>C genotype in healthy individuals would provide information on the associated phenotype. We used SELDI-TOF MS to investigate differentially expressed proteins associated with this *IL6* polymorphism and to determine if pooling serum can be used as an efficient, cost-effective method to select the optimal protein arrays to detect differentially expressed proteins. We also tested whether the *IL6* –174G>C polymorphism was associated with serum IL-6 and HSP60 autoantibody levels in our study population.

2 Methods

2.1 Study population

Specimens were obtained from healthy control participants in an ongoing population-based case-control study of non-Hodgkin lymphoma in the San Francisco Bay Area. Control participants (n = 151) were randomly selected from among those control participants who had enrolled in the study by February 2004 and had provided a blood specimen for molecular and genetic analyses. This study was approved by the University of California, Berkeley, Committee for Protection of Human Subjects.

2.2 DNA isolation

DNA isolation from peripheral blood mononuclear cells was performed with a QIAamp DNA Blood Maxi kit protocol (QIAgen, Santa Clarita, CA, USA), and PicoGreen dsDNA Quantitation kits (Molecular Probes, Eugene, OR, USA) were used according to the manufacturer's instructions to measure the DNA.

2.3 *IL6* –174G>C genotype analysis

IL6 –174G>C analysis was performed using Taqman®-based assays designed by Applied Biosystems (ABI; Foster City, CA, USA). The primer and probe sequences were as follows: 5′-TGACGACCTAAGCTGCACTTTTC (forward primer) and 5′-GGCTGATTGGAAACCTTATTAAGA (reverse primer); TCTTGCGATGCTAAA (VIC probe, G allele) and TCTTGCCATGCTAAA (FAM probe, C allele). Reactions were run on an ABI 7700 under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Genotype was determined by a post-PCR read on the ABI 7700.

2.4 HSP60 antibody ELISA

Analysis of IgG antibodies to HSP60 was performed by ELISA as previously described [13]. Briefly, plates were coated with 0.1 μg/well human recombinant HSP60 (Calbiochem, San Diego, CA, USA). After washing and blocking steps, wells were incubated with serum diluted 1:300 in PBS and a standard curve was made with mouse anti-HSP60 (Calbiochem). Antibody binding was determined with goat anti-human IgG or goat anti-mouse IgG alkaline phosphatase-conjugated antibodies (Calbiochem) and substrate, 0.01 M 4-methylumbelliferylphosphate (Calbiochem) in 0.1 M sodium carbonate buffer (pH 9.8), was measured (excitation 360, emission 460). All samples were run in duplicate and internal controls were included to ensure assay reproducibility.

2.5 Serum IL-6 levels

Serum levels of IL-6 were measured in the same samples analyzed for HSP60 autoantibody levels. An ELISA kit (Biosource International, Camarillo, CA, USA) was used according to the manufacturer's instructions. Briefly, 100 μL of standard or serum was added to wells previously coated with anti-IL-6 antibodies. Following wash steps, incubation with HRP-labeled anti-IL-6 antibodies and addition of the chromogenic solution, the absorbance was read at 450 nm. All standards, samples, and kit controls were run in duplicate.

2.6 Pooled sera

To determine the optimal array surfaces to use in this study, serum pools were prepared based on the presence of the C allele and HSP60 autoantibody levels. One pool consisted of ten samples with the GG genotype and the highest antibody levels and the other pool (*C pool) consisted of ten samples with GC or CC genotypes and the lowest antibody levels (seven GC and three CC). Eighty microliters of each serum sample was pooled and stored in aliquots at −80°C until use. To confirm the effectiveness of pooling serum, 21 individual serum samples were analyzed by CM10 (weak cation exchange) and Q10 (strong anion exchange) arrays. Seven samples from the GG pool, all ten samples in the *C pool and an additional four samples with the CC genotype were analyzed.

2.7 SELDI-TOF MS analysis

Arrays were prepared as described previously [14]. Briefly, CM10 and Q10 arrays were equilibrated with binding buffer (100 mM ammonium acetate (pH 4) or 100 mM sodium ac-

etate (pH 8), respectively). Samples were prepared by adding 180 μL of denaturing buffer (8 M urea, 2% CHAPS, 50 mM Tris, pH 9) to 20 μL of serum, vortexing for 30 min at 4°C and diluting 1:10 in the appropriate binding buffer. Fifteen microliters of sample was added to each spot and incubated for 1 h followed by a series of washes with binding buffer and deionized (DI) H_2O. Sinapinic acid (SPA) was applied and arrays were analyzed by the ProteinChip Reader (Series PBS II, Ciphergen Biosystems, Fremont, CA, USA). IMAC (immobilized metal affinity chromatography) arrays were prepared in the same manner following an initial incubation with copper sulfate and neutralization with sodium acetate. PBS (pH 4) was used as the IMAC binding buffer. NP20 (normal phase) arrays were loaded with 2 μL of sample and allowed to air dry followed by an application of SPA. All samples were run in duplicate and a negative control consisting of binding buffer in place of serum and an internal control consisting of one sample repeated on each array, were added to ensure reproducibility.

2.8 SELDI-TOF MS peak analysis

After baseline subtraction, spectra were normalized and Biomarker Wizard cluster analysis applied with a cluster mass window of 0.8%. The Biomarker Wizard software, which uses the Mann–Whitney test, was used for statistical analysis of individual serum samples and a *t*-test was used for the analysis of pooled sera.

2.9 Apolipoprotein C-I (ApoC-I) purification

GG and *C sera pools were fractionated by anion exchange using Q HyperD F spin columns and buffers provided in the Expression Difference Mapping kit (Ciphergen Biosystems), according to the manufacturer's instructions. Briefly, serum was mixed with denaturing buffer, diluted with U1 buffer, and added to the equilibrated column. Proteins were eluted by the provided buffers of decreasing pH and aliquots of fractions were run on NP20 and CM10 arrays. To obtain a more accurate molecular weight (MW) of the 6.6 and 6.4 kDa proteins, a low MW standard (Ciphergen Biosystems) was applied to the array and used for calibration. The *C flow-through fraction was dried down in a SpeedVac, reconstituted with sample buffer (BioRad, Hercules, CA) and either 100 or 200 μg run on a 16.5% Tris-tricine gel (BioRad) and stained according to the manufacturer's instructions with CBB G-250 (Pierce Biotechnology, Rockford, IL, USA). Two bands near the 6.5 kDa marker were cut out of the gel and incubated for 30 min with 400 μL of 50% methanol and 10% acetic acid followed by 100 μL of ACN for 15 min and an overnight incubation with 20 μL of 50% formic acid, 25% ACN, and 15% isopropanol. Supernatants were run on an NP20 gel and the lower MW band was chosen for identification.

2.10 ApoC-I identification

The gel band was subjected to tryptic digestion. The band was washed with 500 μL of 100 mM ammonium bicarbonate (NH_4HCO_3), followed by a 15 min incubation at 50°C in 150 μL of 100 mM NH_4HCO_3 and 10 μL of DTT. After the addition of 10 μL of 100 mM iodoacetamide and a 15 min incubation, the sample was washed with 500 μL of 50% ACN and 50% 100 mM NH_4HCO_3. Fifty microliters of ACN was added and the sample was dried in a SpeedVac. The sample was incubated with 20 μL of 0.02 μg/μL modified trypsin (Promega, Madison, WI) in 25 mM NH_4HCO_3 for 15 min. Twenty microliters of 25 mM NH_4HCO_3 was added and the sample was incubated overnight at 37°C. The supernatant was saved and additional peptides extracted twice with 50 μL of 60% ACN, 0.1% formic acid.

2.11 MS

A nano-LC column, consisting of 10 cm of Polaris c18 5 μm packing material (Varian, Palo Alto, CA, USA), was packed in a 100 μm inner diameter glass capillary with an emitter tip. The column was directly coupled to an ESI source mounted on a Thermo-Finnigan Decca XP Plus mass spectrometer (Thermo Electron, Waltham, MA, USA). Peptides were eluted on a linear gradient from 0 to 45% at 200 nL/min. Buffer A was 5% ACN, 0.02% heptaflurobutyric acid (HBFA); buffer B was 80% ACN, 0.02% HBFA. SEQUEST was used to identify peptides and proteins from the human protein "Fasta" database [15]. The parent ion and fragment mass tolerance was 1.40 amu with no more than two missed cleavages.

2.12 SELDI-TOF MS immunoassay

PS10 (preactivated) arrays were prepared as described previously [16]. Briefly, arrays were incubated with PBS followed by the addition of Protein G (Calbiochem). Arrays were blocked, washed, and anti-ApoC-I antibody (Abcam, Cambridge, MA, USA) (0.5 mg/mL) was applied and incubated for 1 h. After a series of washes either the denatured serum, prepared as described above, or purified ApoC-I from human plasma (Sigma, St. Louis, MO, USA), was diluted 1:10 in PBS containing 0.5% Triton X-100 (PBS-Triton X) and added to the array for 1 h. The arrays were then washed and 1 μL of CHCA prepared as a saturated solution in 50% ACN and 0.5% TFA was added twice to each spot.

2.13 Statistical analysis

Analyses were restricted to specimens from white non-Hispanic participants ($n = 132$). Specimens with undetectable antibody levels ($n = 5$) and with genotyping failures ($n = 1$) were also excluded. Therefore, the final analyses were based on data from 126 participants. Hardy–Weinberg equilibrium for the *IL6* –174G>C polymorphism was determined using

chi-square analysis. Nonparametric statistical methods were used to compare continuous variables between the groups. All tests of statistical significance were two-sided and considered statistically significant for p-values ≤ 0.05 and somewhat significant for $0.05 < p$-values ≤ 0.10.

3 Results

3.1 *IL6* –174C allele is associated with lower HSP60 autoantibody levels but not with serum IL-6 levels

The mean age of study participants was 58.5 years and ranged from 30 to 84 years. Approximately 59% of participants were men. The *IL6* single nucleotide polymorphism (SNP) was in Hardy–Weinberg equilibrium and the allele frequency of the *IL6* C allele was 0.33. Age and sex were not associated with *IL6* genotype or HSP60 autoantibody levels (data not shown). The C allele was inversely associated with HSP60 autoantibody levels (Table 1); specifically, carriers of the C allele had significantly lower levels of antibodies than noncarriers (29.20 AU/mL *vs.* 48.40 AU/mL, p-value 0.02). The GG genotype had the highest antibody levels (48.40 AU/mL), followed by the GC (37.25 AU/mL), and the CC (23.32 AU/mL). Serum IL-6 was measured to determine the influence of the –174G>C polymorphism. Of the 126 samples tested, only 60 samples had detectable levels of IL-6 (6 CC, 23 GC, and 31 GG). There was no association between IL-6 levels and the –174G>C polymorphism.

3.2 Serum pooled by *IL6* genotype has differences in protein expression

SELDI-TOF MS was used to analyze protein expression on the CM10, Q10, and IMAC protein arrays. Although the Q10 and IMAC analysis did not reveal any differentially expressed proteins, six protein peaks differed between the GG and *C pools on the CM10 array ($p<0.05$). Of these, the 6.4 and 6.6 kDa proteins exhibited 1.8-fold greater expression in carriers of the C allele (Fig. 1).

3.3 Analysis of individual serum samples confirms pooled sera results

There were no significant differences noted in Q10 array analysis of individual samples when analyzed based on the presence of the C allele. However, in the CM10 array analysis three proteins that were differentially expressed between the GG and *C pools were also differentially expressed in the individual analyses ($p<0.05$). Two other proteins significant in the pooled analyses approached significance in the individual analyses (4226 Da $p = 0.069$, 6449 Da $p = 0.056$). Upon comparison of the homozygous genotype groups, the 4226 Da protein was significant ($p<0.01$). Six proteins that were differentially expressed by the presence of the C allele were identified in individual analyses only, including a 7.7 kDa protein down-regulated in C allele carriers ($p = 0.004$). Consistent with the pooled results, 6.6 kDa protein expression was greater among carriers of the C allele compared with the GG genotype (Fig. 2). This protein

Table 1. The *IL6* genotype is associated with HSP60 autoantibody levels

Genotype	*n*	Median age	[HSP60 Ab] (AU/mL)[a] median ± quartiles	*p*-Value[b]	*p*-Value[c]	*p*-Value[d]
IL6						
GG	59	58	48.4 (23.2–106.0)			
GC	51	57	37.2 (16.0–62.2)			
CC	16	57.5	23.3 (18.2–55.2)			
				0.05	0.04	0.03
No C allele	59	58	48.4 (23.2–106.0)			
C allele	67	57	29.2 (17.5–60.3)			
				0.02	0.02	0.02
No G allele	16	57.5	23.3 (18.2–55.2)			
G allele	110	57.5	40.4 (20.8–81.1)	0.10	0.08	0.07
Total	126	57.5	37.0 (19.3–68.7)			

a) AU/mL: Arbitrary units/mL.

b) Kruskal–Wallis test: *IL6* grouped as three genotypes and Wilcoxon two-sample test: *IL6* grouped by C allele using HSP60 antibody concentration (not normally distributed).

c) *F*-test from ANOVA using log transformed HSP60 antibody concentration; Kolmogorov–Smirnov test for normality on log transformed concentration values, $p>0.15$.

d) *F*-test from ANCOVA using log transformed HSP60 antibody concentration and age-group based on tertiles of the distribution (<50, 50–55, >55) as a covariate. Spearman correlation between age (as a continuous variable) and HSP60 antibody concentration, $r = -0.125$, $p = 0.16$.

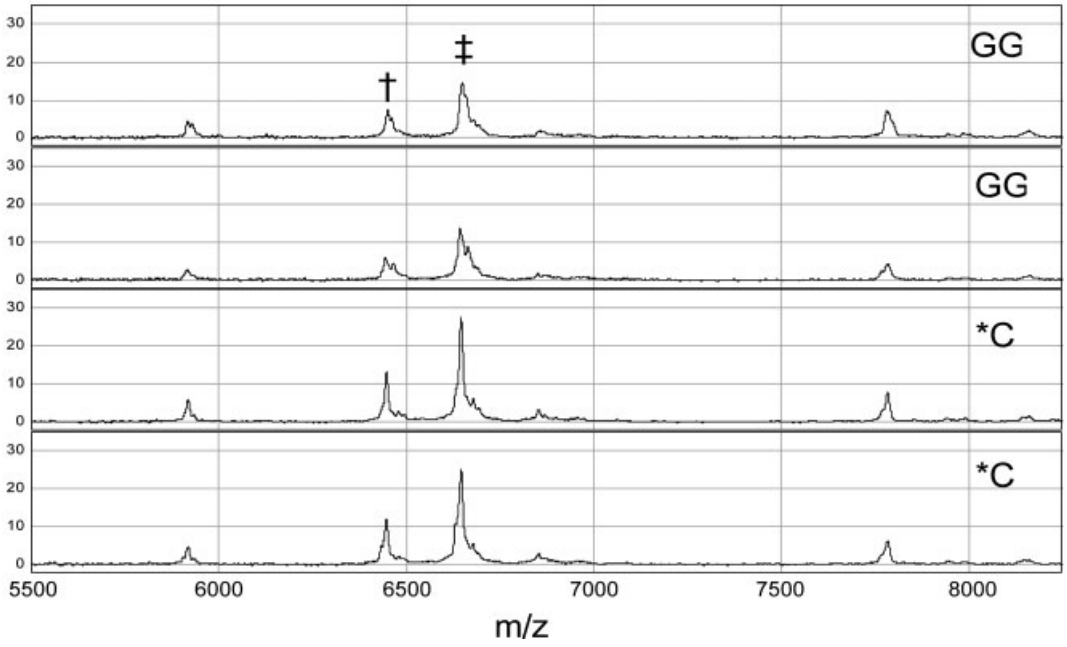

Figure 1. Analysis of pooled sera on a weak cation exchange array. Serum was pooled as described in Section 2 based on the presence of the *IL6*–174C allele and analyzed on a weak cation exchange (CM10) array. Pools consisted of serum from individuals with the GG genotype (GG) or individuals with either the GC or CC genotypes (*C). †, 6.4 kDa protein; ‡ 6.6 kDa protein.

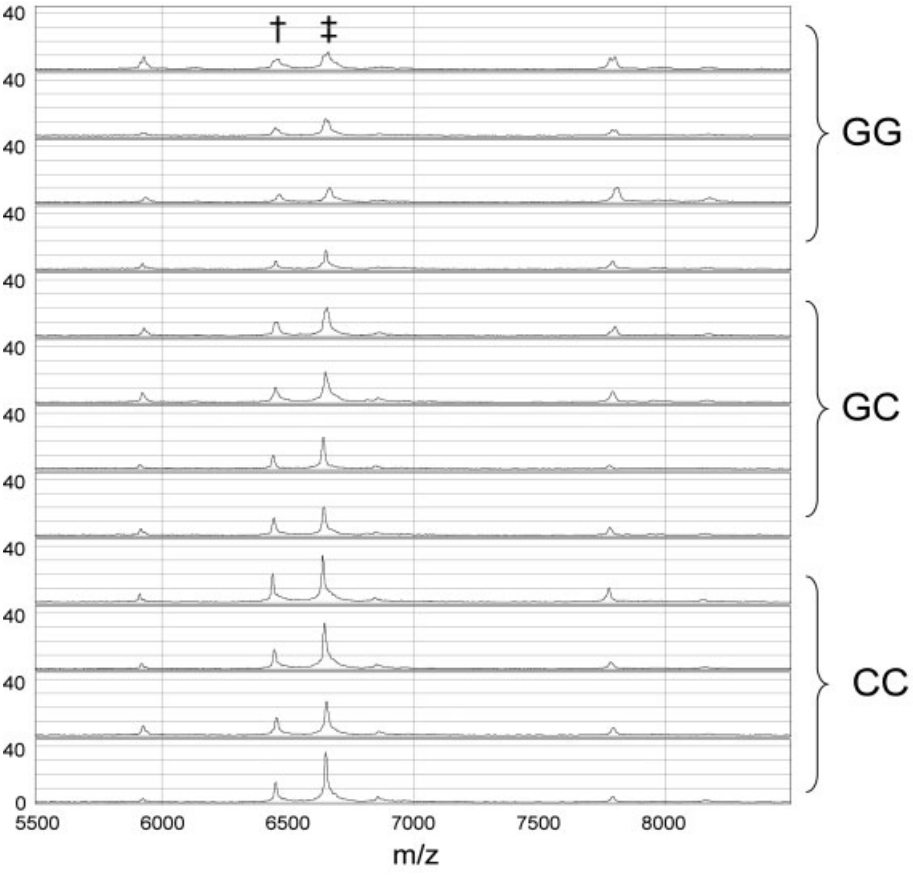

Figure 2. Analysis of individual serum on a weak cation exchange array. Individual serum samples were analyzed on a weak cation exchange (CM10) array. Spectra are from four different individual serum samples *per IL6* genotype (GG, GC, and CC) and are representative of all spectra. †,6.4 kDa protein; ‡, 6.6 kDa protein.

exhibited a gene dose effect with the highest mean intensity among those with the CC genotype, followed by GC, and GG (23.36, 21.64, and 18.66 intensity units, respectively).

3.4 Protein purification and identification

SELDI-TOF MS analysis of anion exchange fractions showed that the flow-through fractions of the GG and *C pooled sera had the greatest abundance of the 6.6 kDa protein and the least amount of albumin (Fig. 3, GG data not shown). Interestingly, the 6.4 kDa protein peak also was in the flow-through fraction. Inclusion of an MW standard on the array to increase mass accuracy revealed more precise MWs of 6624 and 6426 Da for the peaks. The *C flow-through fraction was chosen for identification as it contained greater amounts of the peak of interest. Analysis of the fraction on a Tris-tricine gel showed a band next to the 6.5 kDa aprotinin marker and another faint band corresponding to a slightly smaller MW (data not shown). Extraction of protein from both the bands and analysis on an NP20 array revealed an 8.8 kDa upper band, while the lower band contained the 6.6 and 6.4 kDa proteins as well as a slight contamination of the 8.8 kDa protein (Fig. 4).

SEQUEST analysis of the tryptic peptides from the band containing the 6.6 and 6.4 kDa proteins identified three peptides from the ApoC-I precursor (Swiss-Prot number P02654) with a 54.4% sequence coverage for the processed protein with an MW of 6630 Da. The amino acid sequence of the processed ApoC-I is as follows: TPDV SSALDK*LKEF GNTLED-KARE* LISR*IKQSEL SAK***MREWFSE TFQK*VKEKLK IDS.

The three identified peptides are underlined with an asterisk separating two consecutive peptides. Previous studies have identified a truncated ApoC-I protein missing the N-terminal threonine and proline (a 198 Da difference) with an MW of 6432 Da [17, 18]. As mentioned previously, we determined the MWs of the 6.6 and 6.4 kDa peaks to be 6624 and 6426 Da, which is a difference of 198 Da. Further, a correlation analysis of peak intensities from the individual serum analysis shows a correlation coefficient of 0.83 between the 6426 and 6624 Da peaks as a further indication that the 6.4 kDa peak is ApoC-I. Additionally, we have tentatively identified the 7.7 kDa protein as platelet factor (PF)4 based

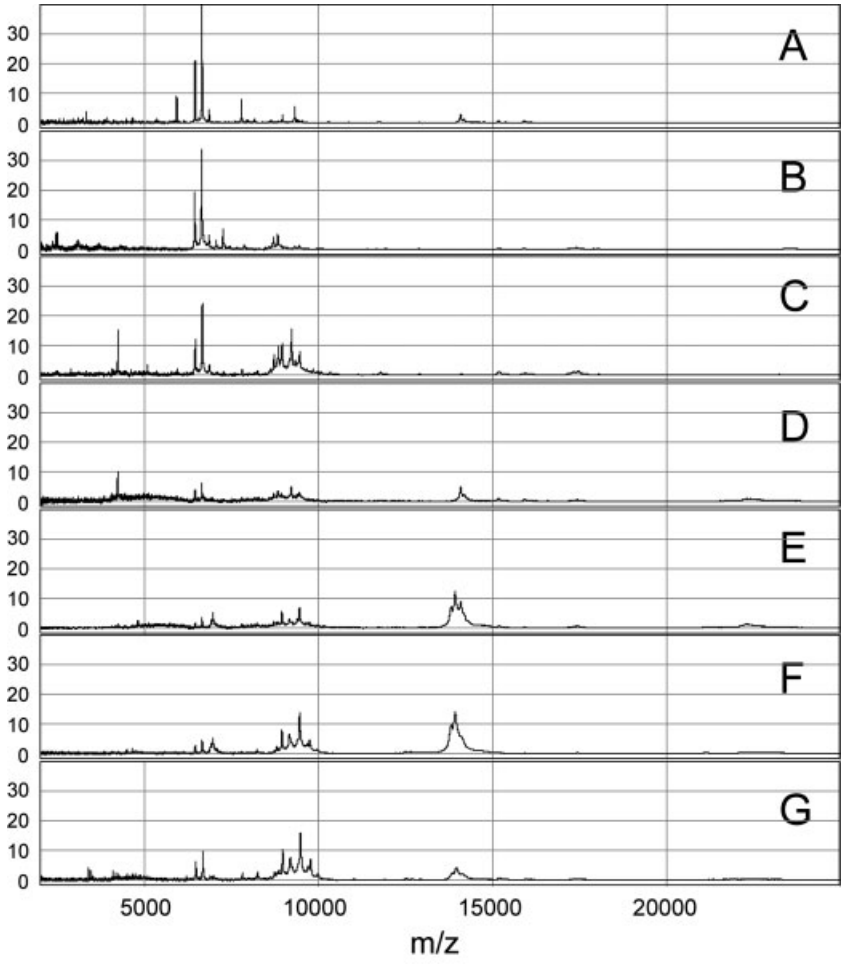

Figure 3. Anion exchange fractionation of pooled sera. Serum was pooled as described in Section 2. The pool containing serum from individuals with the GC or CC genotype (*C) was fractionated by anion exchange and the flow-through was analyzed on a weak cation exchange (CM10) array while all other fractions were analyzed on normal phase (NP20) arrays. (A) Flow-through, (B) pH 9, (C) pH 7, (D) pH 5, (E) pH 4, (F) pH 3, and (G) organic wash.

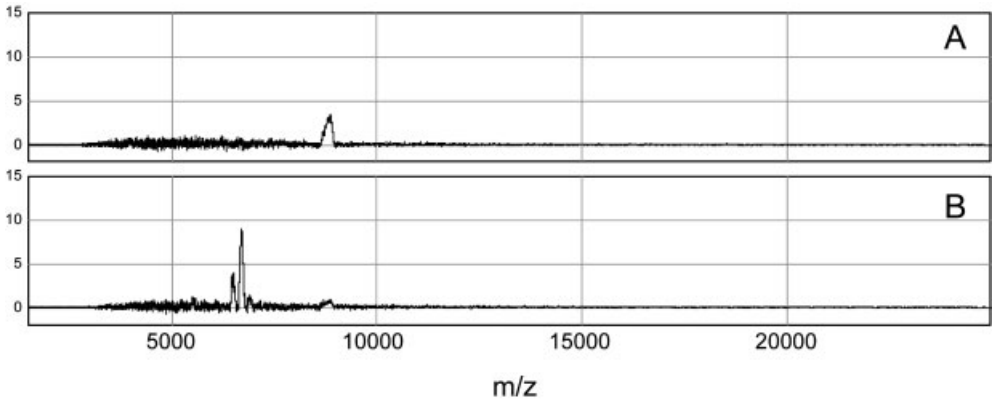

Figure 4. Analysis of proteins purified from the Tris-tricine gel. The flow-through fraction of the pooled sera from individuals with either the GC or CC genotype (*C) was analyzed on a Tris-tricine gel. Protein was extracted from two bands cut from the gel and run on a normal phase (NP20) array. (A) Upper gel band with an MW of 8.8 kDa. (B) Lower band with MWs of 6.4 and 6.6 kDa and some residual 8.8 kDa protein.

on the observation of nonspecific binding during the ApoC-I immunoassay, which had previously been used to identify PF4 in our laboratory [19], as well as its MW and p*I*.

3.5 Confirmation of ApoC-I through SELDI-TOF MS immunoassay

A SELDI-TOF MS immunoassay was performed to confirm the identity of the 6.6 and 6.4 kDa proteins as ApoC-I. Analysis of the pooled sera showed two clear peaks at 6.6 and 6.4 kDa, as well as a minor peak at 3.3 kDa, that represent the full length ApoC-I protein, the shortened ApoC-I, and the full length ApoC-I +2 charged species, respectively (Fig. 5). Compared with the control ApoC-I purified from human plasma, these peaks confirm the identity of the 6.6 and 6.4 kDa protein as ApoC-I. The accuracy of the antibody assay was confirmed through analysis of the proper controls including the use of a nonspecific antibody.

4 Discussion

The IL-6 proinflammatory cytokine plays an important role in the immune system and the –174G>C promoter polymorphism is associated with multiple disease states [8, 20, 21]. However, the reported function of the polymorphism and its effects on circulating IL-6 levels are inconsistent between studies. In this study, SELDI-TOF MS was used to examine the association between phenotypic changes in the human serum proteome of healthy individuals and the *IL6* –174G>C promoter polymorphism. We show that this polymorphism is associated with differences in the human serum proteome and that the C allele is associated with increased serum ApoC-I. Additionally, our results corroborate earlier reports of an association between the *IL6* –174C allele and lower HSP60 autoantibody levels, and of no association between this *IL6* genotype frequency and serum IL-6 levels [9].

Results from studies of the effect of the *IL6* –174G>C polymorphism on circulating IL-6 levels have been inconsistent. *In vitro* studies, as well as measurements of plasma levels in adults, showed a significant association of the –174C allele with lower basal IL-6 levels [7]. However, a separate study reported that the CC genotype was associated with greater IL-6 production in neonatal cord blood and neonatal mononuclear cells, but not adult plasma levels [22]. Consistent with our results, other studies have also found no association between genotype and IL-6 levels [9, 12]. Only a limited number of subjects had detectable serum IL-6 levels which is consistent with results reported by Biosource International (the manufacturer of the ELISA kit) and earlier studies [9]. The half-life of IL-6 in plasma is less than 2 h [23] and is likely to have contributed to the limited number of samples with detectable IL-6 levels in our and other investigators' studies. Therefore, studies designed to accommodate the 2-h test window are needed to assess more accurately whether the *IL6* –174G>C polymorphism is associated with IL-6 levels and if associations between the polymorphism and disease states are a direct or indirect result of IL-6.

The –174C allele has been inversely associated with young adult Hodgkin lymphoma and young adult nodular lymphocyte predominant Hodgkin lymphoma [24, 25]. Additionally, Kaposi sarcoma, which is associated with an increased risk for non-Hodgkin lymphoma [26, 27] and systemic-onset juvenile chronic arthritis, has been associated with elevated levels of inflammatory cytokines including IL-6, IL-1, and TNF-α [28–32]. In turn, clinical studies showed elevated IL-6 and IL-1 levels among Hodgkin lymphoma patients and non-Hodgkin lymphoma is associated with various autoimmune and chronic inflammatory disorders [33–35]. Combined, these results support a model where the C

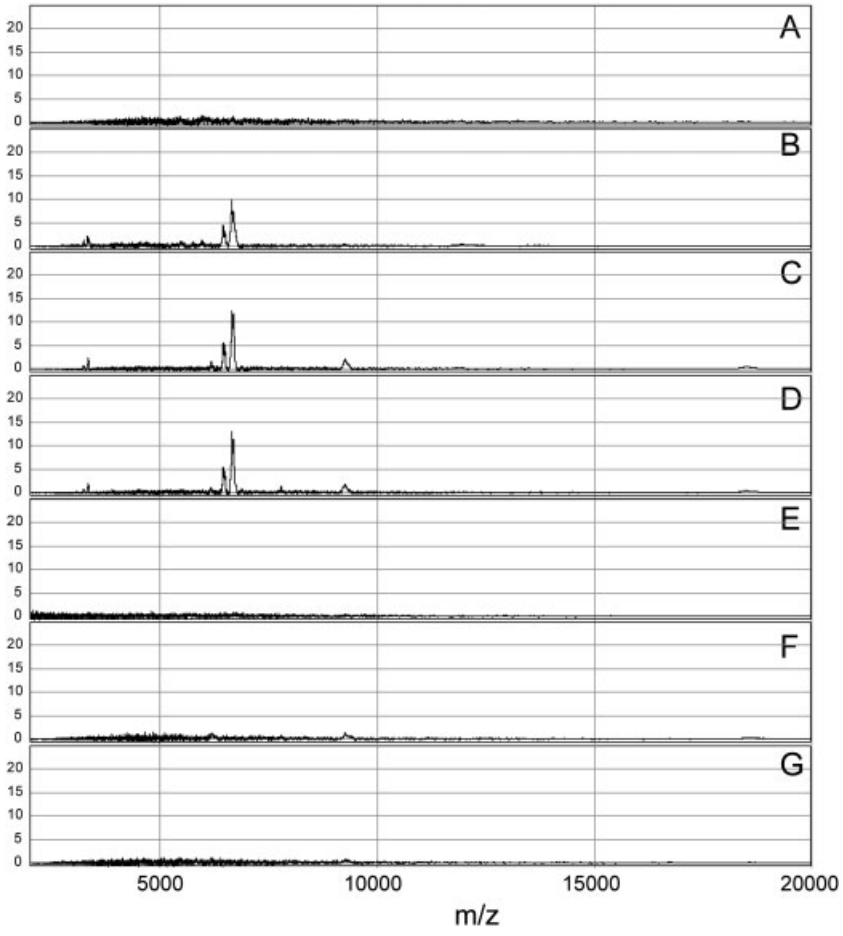

Figure 5. SELDI-TOF immunoassay of ApoC-I. Preactivated (PS10) arrays were coated with protein G and anti-ApoC-I antibody followed by incubation with purified ApoC-I or pooled sera. (A) No ApoC-I, (B) purified ApoC-I, (C) pooled sera from individuals with the GG genotype, (D) pooled sera from individuals with the GC or CC genotype, (E) no antibody, purified ApoC-I, (F) nonspecific antibody, pooled sera from individuals with the GC or CC genotype, and (G) no antibody, pooled sera from individuals with the GC or CC genotype.

allele confers protection for inflammatory-related diseases. Given that IL-6 signaling leads to proliferation of T-cells [4, 5] and increased immunoglobulin secretion in antigen-stimulated B-cells [36], our results that show lower HSP60 autoantibody levels associated with the C allele provide further support that the *IL6* –174 C allele is associated with decreased downstream effects of IL-6 activity and inflammation.

In this study we report an association between the *IL6* –174C allele and increased serum ApoC-I levels. ApoC-I is a component of triglyceride-rich lipoproteins known to decrease the clearance of lipoproteins through the low-density lipoprotein (LDL) receptor, the LDL receptor-related protein (LRP), and the very low-density lipoprotein (VLDL) receptor. Recent studies have focused on ApoC-I-mediated inhibition of plasma cholesteryl ester transfer protein (CETP) [37–42]. CETP transfers cholesterol esters from high-density lipoprotein (HDL) to LDL and VLDL, and its inhibition leads to increased HDL and decreased LDL levels [41–43] that may play a role in inflammation. HDL has antioxidant and anti-inflammatory properties such as the paraoxonase-mediated breakdown of oxidized phospholipids in LDL, which can stimulate cytokine expression as well as monocyte recruitment and adhesion [44]. Inflammation is associated with reduced HDL levels [45–47] and decreased anti-inflammatory and antioxidant enzymes, as well as increased proinflammatory factors such as lipid oxidation within HDL [48]. Therefore, an *IL6* –174C phenotype associated with elevated serum ApoC-I and low HSP60 antibody levels may favor an anti-inflammatory state that confers protection against proinflammatory conditions such as systemic-onset juvenile chronic arthritis and Kaposi sarcoma. An additional protein at 7.7 kDa, which was significantly down-regulated among carriers of the *IL6* –174C allele has been tentatively identified as PF4. PF4 is a platelet derived proinflammatory CXC-chemokine that was previously identified by our laboratory [19] in a study of benzene-exposed workers. Raised plasma levels of PF4 have been observed in individuals with hypercholesterolemia, inflammatory bowel disease and other proinflammatory conditions [49, 50]. Thus, the putative inverse association between PF4 levels and the *IL6* C allele lends further credence to an anti-inflammatory role for the *IL6* C allele. Studies to confirm the identification of the PF4 protein are currently underway.

In conclusion, we have examined phenotypic differences in the human serum proteome associated with the *IL6* –174G>C polymorphism and found an association between

the C allele and increased ApoC-I that may clarify the protective effect of IL-6 on various diseases. Additionally, we have corroborated previous findings of an association with the C allele and decreased HSP60 autoantibody levels. In addition, using SELDI-TOF MS, we have shown that pooling sera can be useful to determine the ideal protein array surface for the analysis of differential protein expression, especially in epidemiological studies where resources may be limited. Further studies are needed to investigate the association between the *IL6* –174G>C polymorphism and ApoC-I and to examine its role in disease susceptibility.

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6 Addendum

Following acceptance of this manuscript, a study was published reporting an inverse association between the *IL6* CC genotype and LDL levels (n=252) further substantiating the anti-inflammatory role of the *IL6*–174C allele. This association remained for five years after follow-up.
Henningsson S., Hakansson A., Westberg L., Baghaei F. *et al.*, *Obesity* 2006 *14*, 1868–1873.

