Relationship Among Circulating Inflammatory Proteins, Platelet Gene Expression, and Cardiovascular Risk

David D. McManus, Lea M. Beaulieu, Eric Mick, Kahraman Tanriverdi, Martin G. Larson, John F. Keaney, Emelia J. Benjamin, Jane E. Freedman

Objective—Cardiovascular disease is a complex disorder influenced by interactions of genetic variants with environmental factors. However, there is no information from large community-based studies examining the relationship of circulating cell–specific RNA to inflammatory proteins. In light of the associations among inflammatory biomarkers, obesity, platelet function, and cardiovascular disease, we sought to examine the relationships of C-reactive protein (CRP) and interleukin-6 (IL-6) to the expression of key inflammatory transcripts in platelets.

Approach and Results—We quantified circulating levels of CRP and IL-6 in 1625 participants of the Framingham Heart Study (FHS) Offspring cohort examination 8 (mean age, 66.6±6.6 years; 46% men). We measured the expression of 15 relevant genes by high-throughput quantitative reverse transcriptase polymerase chain reaction from platelet-derived RNA and used multivariable regression to relate serum concentrations of CRP and IL-6 with gene expression. Levels of CRP and IL-6 were associated with 10 of the 15 platelet-derived inflammatory transcripts, ALOX5, CRP, IFIT1, IL6, PTGER2, S100A9, SELENBP1, TLR2, TLR4, and TNFRSF1B (P<0.001). Associations between platelet mRNA expression with CRP and IL-6 persisted after multivariable adjustment for potentially confounding factors. Six genes positively associated with CRP and IL-6 in the FHS sample were also upregulated in megakaryocytes in response to CRP and IL-6 exposure.

Conclusions—Our data highlight the strong connection between the circulating inflammatory biomarkers CRP and IL-6 and platelet gene expression, adjusting for cardiovascular disease risk factors. Our results also suggest that body weight may directly influence these associations. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: XXX

Human genome-wide association studies have led to better understanding of the genetic basis of complex cardiovascular diseases (CVD) and risk factors.1,2 In contrast, few gene expression profiling studies have been reported from large community-based cohorts.3,4 Quantitative differences in the relative abundance of transcripts contribute to susceptibility to, and prognosis of, human diseases, including CVD.5,6 Transcript expression is heritable, and genetic expression analyses provide insights into the pathogenesis of disease based on the strong relationships among DNA, RNA, and proteins.5 The emerging literature suggests that levels of gene expression diverge between individuals with and without CVD and its risk factors (eg, obesity, high-density lipoprotein concentrations).7-14

Inflammatory cytokines, most notably C-reactive protein (CRP), have generated considerable interest as biomarkers to risk stratify and guide therapy for individuals at risk for CVD.15-20 In addition to predicting CVD, CRP may directly facilitate atherogenesis and thrombosis.21,22 Interleukin 6 (IL-6), another inflammatory cytokine, through IL-6 receptor triggers the production of CRP and other acute-phase reactants.23 Importantly, IL-6 production occurs not only in hepatocytes but also in adipose tissue.24 Multiple recent large studies have demonstrated that specific genetic mutations in the IL-6/IL-6 receptor pathway, leading to less IL-6 signaling, were found to lower coronary heart disease risk.24,25

Based on previous in vitro and animal work, we selected 15 platelet-derived genes whose expression may relate to circulating IL-6 or CRP.7,26-37 Our primary hypothesis was that the circulating inflammatory proteins IL-6 and CRP are associated with platelet transcripts and are modulated by obesity.

Received on: January 10, 2013; final version accepted on: August 9, 2013.

From the National Heart Lung and Blood Institute’s and Boston University’s Framingham Heart Study, Framingham, MA (D.D.M., K.T., M.G.L., J.E.F.); Cardiology Division, Department of Medicine (D.D.M, L.M.B., K.T., J.F.K., J.E.F.) and Epidemiology Division, Department of Quantitative Health Sciences (D.D.M, E.M.), University of Massachusetts Medical School, Worcester, MA; Section of Cardiovascular Medicine, Department of Medicine (E.J.B.) and Department of Mathematics and Statistics (M.G.L.), Boston University, Boston, MA; Preventive Medicine Section, Department of Medicine, Boston University School of Medicine, Boston, MA (E.J.B.); and Department of Epidemiology (E.J.B.) and Department of Biostatistics (M.G.L.), Boston University School of Public Health, Boston, MA.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.orglookup/suppl/doi:10.1161/ATVBHA.112.301112/-/DC1. Correspondence to David D. McManus, MD, MSc, Cardiology Division, University of Massachusetts Medical School, Worcester, MA 01655. E-mail mcmanusd@ummc.org

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBHA.112.301112
Materials and Methods

Clinical, demographic, and laboratory characteristics of the 1625 FHS Offspring cohort 8 participants in our analysis are shown in Table 1. We studied middle-aged to older adults (67±7 years; 46% men) with a modest prevalence of CVD risk factors. After adjustment for potentially confounding variables, circulating levels of ln-transformed CRP and IL-6 were significantly associated with 8 of 15 platelet-derived genes (Table 2). Regression models including all covariates in adjusted models and fitted with CRP or IL-6 as the dependent variable, as well as platelet gene expression, are shown in Table I in the online-only Data Supplement. Arachidonate 5-lipoxygenase (ALOX5), PTGER2, S100A9, SELENBP1, TLR4, and TNFRSF1B expression was higher with greater mean levels of ln-transformed CRP and IL-6. In addition, higher mean levels of circulating CRP were also associated with greater CD163 and NFKB1 expression. Increased circulating IL-6 was negatively associated with CRP gene expression but positively associated with TLR2 gene expression.

We found evidence of an interaction between obesity (as defined by a body mass index ≥30 kg/m²) and ALOX5, NFKBIA, SELENBP1, and SIRPA gene expression for IL-6 and NFKBIA and SIRPA for CRP (Figure 1). We did not observe an interaction between gene expression and inflammatory biomarkers in overweight or normal weight individuals. In an effort to better understand the associations among obesity, inflammatory cytokines, and platelet gene expression, we related levels of several circulating biomarkers associated with either body mass index or circulating adipocytokines, including intercellular adhesion molecule 1, monocyte chemoattractant protein-1, P-selectin, and tumor necrosis factor receptor. Adjustment for circulating adipose-related factors did not substantially alter observed associations between CRP and IL-6 and platelet gene expression (Table II in the online-only Data Supplement).

Gene expression was measured in human megakaryocytes after exposure to IL-6, CRP, or both, using concentrations reported in obese patients and individuals with CVD based on basic and clinical reports. Treatment of human megakaryocytes with 20 µg/mL of CRP resulted in a reduction in ALOX5 expression (Table 3). Exposure of these cells to 10 pg/mL of IL-6 resulted in an increase in CD163, IFIT1, IL-6, NFKBIA, and TBFRSF1B expression. Combined treatment with IL-6 and CRP did not significantly alter any of the transcripts examined.

To explore how megakaryocytes responded to different concentrations of IL-6 and CRP exposure, we measured platelet mRNA levels in wild-type mouse megakaryocyte cells treated with 3 different concentrations of IL-6 or CRP. As seen in human megakaryocytes, levels of NFKB1 were significantly increased in mouse megakaryocytes exposed to IL-6 (Table IIIA and IIIB in the online-only Data Supplement). The overall response of mouse megakaryocytes to CRP and IL-6 exposure was distinct from that observed in human cells. Notably, immunofluorescence staining of mouse megakaryocytes for NFkBIA, a transcript upregulated by both IL-6 and CRP in these cells, showed little to no change in NFkBIA protein levels (Figure 2 and Figure IA and IB in the online-only Data Supplement).

Recent work has shown that interleukin-1β exposure alters protein levels in mature human platelets. We saw no statistically significant changes to levels of key inflammatory transcripts in human platelets treated with IL-6 and CRP versus untreated platelets (Table IVA and IVB in the online-only Data Supplement). To explore the possibility that protein levels are altered without changes to mRNA levels, we used Western blots and immunofluorescence to quantify levels of a representative inflammatory cytokine, TNFRSF1B, in human platelets exposed to CRP and IL-6. As seen in Figure 3A and 3B, the total protein levels of TNFRSF1B were increased in treated platelets versus referent platelets. The increase in TNFRSF1B was not caused by any contamination of immune cells because there was no signal for CD45 (Figure IC in the online-only Data Supplement), a transmembrane glycoprotein expressed by multiple immune cells. TNFRSF1B is expressed
Table 2. Association Between Gene Expression and CRP or IL-6 Levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name</th>
<th>Function</th>
<th>IL-6, pg/mL</th>
<th>CRP, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Coeff±SE P Value</td>
<td>Coeff±SE P Value</td>
</tr>
<tr>
<td>ALOX5</td>
<td>Arachidonate 5-Lipoxygenase</td>
<td>Inflammation</td>
<td>0.012±0.005 0.01*</td>
<td>0.020±0.008 0.01*</td>
</tr>
<tr>
<td>CD163</td>
<td>Hemoglobin scavenger receptor</td>
<td>Inflammation</td>
<td>0.008±0.005 0.11</td>
<td>0.018±0.008 0.02*</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
<td>Inflammation</td>
<td>−0.009±0.004 0.01*</td>
<td>−0.012±0.006 0.05</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecule 1</td>
<td>Adhesion</td>
<td>0.009±0.005 0.06</td>
<td>0.010±0.008 0.20</td>
</tr>
<tr>
<td>IFIT1</td>
<td>Interferon-induced protein with tetratricopeptide repeats 1</td>
<td>N/A</td>
<td>0.008±0.004 0.06</td>
<td>0.008±0.007 0.24</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
<td>Inflammation</td>
<td>−0.006±0.004 0.15</td>
<td>−0.009±0.007 0.20</td>
</tr>
<tr>
<td>NFKB1</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B cells 1</td>
<td>Inflammation</td>
<td>0.010±0.005 0.05</td>
<td>0.020±0.008 0.01*</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>Nuclear factor -κB inhibitor α</td>
<td>Inflammation</td>
<td>0.006±0.005 0.22</td>
<td>0.005±0.007 0.46</td>
</tr>
<tr>
<td>PTGER2</td>
<td>Prostaglandin E receptor 2 (subtype EP2)</td>
<td>Inflammation</td>
<td>0.012±0.005 0.01*</td>
<td>0.022±0.008 0.006*</td>
</tr>
<tr>
<td>S100A9</td>
<td>S100 calcium-binding protein A9</td>
<td>Inflammation</td>
<td>0.020±0.005 &lt;0.001*</td>
<td>0.029±0.008 &lt;0.001*</td>
</tr>
<tr>
<td>SELENBP1</td>
<td>Selenium-binding protein 1</td>
<td>N/A</td>
<td>0.013±0.004 0.003*</td>
<td>0.022±0.007 0.003*</td>
</tr>
<tr>
<td>SIRPA</td>
<td>Signal-regulatory protein α</td>
<td>Inflammation</td>
<td>0.006±0.004 0.13</td>
<td>0.011±0.007 0.13</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
<td>Inflammation</td>
<td>0.011±0.004 0.008*</td>
<td>0.012±0.007 0.06</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
<td>Inflammation</td>
<td>0.017±0.005 &lt;0.001*</td>
<td>0.019±0.008 0.02*</td>
</tr>
<tr>
<td>TNFRSF1B</td>
<td>Tumor necrosis factor receptor superfamily 1B</td>
<td>Inflammation</td>
<td>0.012±0.005 &lt;0.001*</td>
<td>0.022±0.008 0.004*</td>
</tr>
</tbody>
</table>

These are from models correcting for the clinical confounders listed in the Methods section using the log-transformed assay levels. Coeff indicates estimated regression coefficient.

*Statistically significant relationship correcting for the number of statistical tests and using false discovery rate of 5%. Functions for IFIT1 and SELENBP1 are not well characterized.

Discussion

Although studies have previously shown associations between CVD and inflammatory biomarkers, the interaction with blood-derived gene expression in a large population has never been reported. In this community-based study involving 1625 FHS Offspring cohort 8 participants, we sought to examine the relationship between inflammatory cytokines CRP and IL-6 with inflammatory-related transcripts in platelets. We chose to study platelets both because of our previous data demonstrating an association with obesity and the increased relevance of specifically studying a cell that directly contributes to CVD. Of the 15 transcripts, 6 positively correlated with both CRP and IL-6 and 6 were upregulated or downregulated significantly in megakaryocytes exposed to one of the 2 cytokines. Previously, all of these platelet transcripts were associated with higher mean body mass index.7 Two platelet transcripts were specific to each cytokine. TLR2 expression was positively correlated with IL-6, whereas the level of platelet-derived TLR2 was inversely related to circulating levels of CRP. The expression of CD163 and NFKB1 was positively related to CRP levels but not IL-6 levels.

Previously, cohort studies have demonstrated associations among IL-6, CRP, and CVD, including coronary heart disease and sudden death in men and women.49–52 Relevant to our platelet findings, high plasma IL-6 also has been associated with increased coronary stent thrombosis, suggesting that it may be involved in clinically relevant CVD outcomes.53 Despite these observations, little is known about the mechanisms linking circulating inflammatory markers and platelet function.

Platelets are anucleated and receive all their RNA from megakaryocytes, their precursor cells. Megakaryocytes are found primarily in the bone marrow and may respond to circulating inflammatory cytokines, thereby altering the RNA profile of platelets. As seen our in vitro work, megakaryocytes exposed...
to CRP and IL-6 resulted in altered expression of genes examined in our community-based investigation. We observed that 6 genes associated with circulating IL-6 and CRP levels in our FHS analyses were also significantly upregulated or downregulated in the human megakaryocytes exposed to these factors. Although treatment of mouse megakaryocytes with IL-6 and CRP did not result in statistically significant changes in all 6 of these genes (with the exception of \textit{NFKB1}), we did observe a dose-dependent and directionally similar effect on levels of 4 of the 6 genes altered in human megakaryocytes. From these data, we cannot deduce whether this difference is as a result of species or some other factor.

There were, however, some inconsistencies between our observations in the platelet and megakaryocyte in vitro work and our FHS data. The discrepant findings may be because of the fact that the intensity and duration of exposure to CRP or IL-6 could not be quantified in vivo and thus may have differed from our in vitro experiment. This hypothesis is supported by our mouse experiments, showing that different levels of CRP and IL-6 elicit differential transcriptional responses. There are also inherent differences between native megakaryocytes and megakaryoblastic cell lines. As has been previously shown\textsuperscript{54} and was observed in our experiments, differences in platelet transcripts between humans and mice could have also contributed to discrepant findings. Another possibility, supported by previous work and our finding that levels of \textit{TNFRSF1B} were altered in human platelets in response to CRP and IL-6, is that mature platelets, not megakaryocytes, may directly respond to circulating IL-6 and CRP. Nevertheless, our findings overall support the hypothesis that inflammatory cytokines, such as IL-6 and CRP, may alter the course of megakaryocyte development and thereby affect platelet gene expression. Levels of platelet-derived proinflammatory transcripts may directly influence platelet function or may transfer RNA to other cell types, including endothelial cells.\textsuperscript{34,55}

We observed an interaction between obesity status and the associations of IL-6 and CRP with levels of 4 platelet-derived gene transcripts. One of the genes, \textit{ALOX5}, encodes a lipoxygenase responsible for transforming essential fatty acids into leukotrienes, which exert proinflammatory effects, including induction of nuclear factor (NF)-\textkappa B and IL-6 secretion by adipose tissue.\textsuperscript{56–58} We observed that platelet \textit{ALOX5} expression was higher among participants with elevated IL-6 and

### Table 3. Effects of CRP and IL-6 on Meg-01 (Human Megakaryocyte) Gene Expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>20 µg/mL CRP ΔCt±SE</th>
<th>Fold Change</th>
<th>10 pg/mL IL-6 ΔCt±SE</th>
<th>Fold Change</th>
<th>CRP and IL-6 ΔCt±SE</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALOX5</td>
<td>2.69±1.11*</td>
<td>0.15</td>
<td>1.11±1.11</td>
<td>0.46</td>
<td>−0.84±1.22</td>
<td>1.79</td>
</tr>
<tr>
<td>CD163</td>
<td>1.45±0.67</td>
<td>0.37</td>
<td>−2.77±0.82**</td>
<td>6.82</td>
<td>−1.47±0.67</td>
<td>2.78</td>
</tr>
<tr>
<td>CRP</td>
<td>1.19±0.87</td>
<td>0.43</td>
<td>−1.60±0.97</td>
<td>3.03</td>
<td>−0.77±0.79</td>
<td>1.69</td>
</tr>
<tr>
<td>ICAM1</td>
<td>0.17±1.21</td>
<td>0.89</td>
<td>−1.06±1.21</td>
<td>2.09</td>
<td>−1.62±0.99</td>
<td>3.07</td>
</tr>
<tr>
<td>IFIT1</td>
<td>−0.43±1.19</td>
<td>1.35</td>
<td>−3.05±1.19*</td>
<td>8.32</td>
<td>−1.28±0.97</td>
<td>2.43</td>
</tr>
<tr>
<td>IL6</td>
<td>0.03±0.58</td>
<td>0.97</td>
<td>−2.92±0.58***</td>
<td>7.59</td>
<td>−0.59±0.47</td>
<td>1.51</td>
</tr>
<tr>
<td>NFKB1</td>
<td>−0.06±0.95</td>
<td>1.04</td>
<td>−2.51±0.95**</td>
<td>5.69</td>
<td>−0.56±0.77</td>
<td>1.48</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>−0.86±0.89</td>
<td>1.82</td>
<td>−2.11±0.89</td>
<td>4.32</td>
<td>0.56±0.89</td>
<td>0.67</td>
</tr>
<tr>
<td>PTGER2</td>
<td>0.01±1.19</td>
<td>0.99</td>
<td>−2.18±1.19</td>
<td>3.54</td>
<td>−0.61±0.98</td>
<td>1.52</td>
</tr>
<tr>
<td>S100A9</td>
<td>1.81±1.24</td>
<td>0.28</td>
<td>−0.48±1.24</td>
<td>1.40</td>
<td>−0.37±1.24</td>
<td>1.29</td>
</tr>
<tr>
<td>SELRB1</td>
<td>1.01±2.64</td>
<td>0.49</td>
<td>2.62±2.15</td>
<td>0.16</td>
<td>−1.17±2.64</td>
<td>2.25</td>
</tr>
<tr>
<td>SIRPA</td>
<td>1.63±1.31</td>
<td>0.32</td>
<td>−1.20±1.31</td>
<td>2.30</td>
<td>−1.04±1.31</td>
<td>2.06</td>
</tr>
<tr>
<td>TLR2</td>
<td>2.42±1.46</td>
<td>0.19</td>
<td>0.24±1.46</td>
<td>0.85</td>
<td>−1.09±1.46</td>
<td>2.12</td>
</tr>
<tr>
<td>TLR4</td>
<td>−1.22±1.35</td>
<td>2.34</td>
<td>−2.39±1.35</td>
<td>5.26</td>
<td>−1.69±1.30</td>
<td>3.22</td>
</tr>
<tr>
<td>TNFRSF1B</td>
<td>−2.5±1.32</td>
<td>5.69</td>
<td>−3.43±1.33*</td>
<td>10.82</td>
<td>−0.82±1.08</td>
<td>1.77</td>
</tr>
</tbody>
</table>

CRP indicates C-reactive protein; and IL-6, interleukin 6.

\*P<0.05, **P<0.01, ***P<0.001 compared with no treatment set to 1.00.

---

Figure 2. Effects of interleukin-6 (IL-6) and C-reactive protein (CRP) on NFxBIA protein levels in mouse megakaryocytes. Mouse bone marrow was treated with IL-6 or CRP for 24 hours. Permeabilized samples were stained for CD41–fluorescein isothiocyanate (a megakaryocyte marker) and NFxBIA with a Texas Red–labeled secondary antibody. Isotype controls are shown in Figure IA in the online-only Data Supplement.
CRP levels after adjustment for several potential confounding factors. Obesity did not seem to modulate the interaction between ALOX5 and CRP, but the association between circulating IL-6 and ALOX5 expression was influenced by obesity status. In vitro, megakaryocyte production of ALOX5 was significantly decreased in the presence of CRP at concentrations reported in obese individuals. The discrepancy may be the result of the amount of time the megakaryocytes were exposed to these inflammatory cytokines or to the concentrations used. Taken together, these data suggest that obesity plays a role in influencing ALOX5 transcript levels, perhaps via circulating CRP and IL-6.

NFKB1 encodes NF-κB, a transcription factor that is activated by intracellular and extracellular cytokines. Activated NF-κB stimulates the expression of genes involved in a wide variety of biological functions, including inflammation and thrombosis. NFKB1 expression was positively related to CRP but not IL-6 levels, and there was no interaction between NFKB1 expression and obesity status. However, expression of the gene NFKB1A, which encodes a member of a family of proteins that inhibit NF-κB, was significantly higher in obese individuals in relation to both IL-6 and CRP levels. In vitro, it was only significantly upregulated by IL-6 in megakaryocytes. NFKB1A would result in sequestration of NF-κB in an inactivated state and would block the ability of NF-κB to bind to DNA. Increased expression of this inhibitory protein in response to IL-6 and CRP in obese individuals is notable but of unclear biological significance.

SELENBP1 encodes selenium-binding protein 1. Selenium is an essential nutrient, and selenium deficiency has been associated with certain malignancies. Although the role of SELENBP1 and its significance to CVD remain unclear, we observed that SELENBP1 was strongly and directly related to circulating IL-6 and CRP. We also noted that obesity significantly modulated the interaction between SELENBP1 and IL-6. There was an increase in the expression of SELENBP1 in the megakaryocytes in the presence of both IL-6 and CRP, but it was not significant. Further studies are warranted to define the effects of selenium and selenium-binding protein 1 on platelet function and atherogenesis.

SIRPA encodes signal-regulatory protein-α, a protein involved in regulating monocyte adhesion, migration of monocytes across the endothelial wall, and phagocytosis. Signal-regulatory protein-α may serve a key role in preventing excess monocyte collection and activation of monocytes in the arterial wall during the formation of atherosclerotic plaques. As such, it is notable that, although SIRPA expression was not significantly associated with IL-6 or CRP levels, platelet expression of SIRPA was markedly higher in patients with obesity in response to these inflammatory biomarkers. Our in vitro experiment also showed that megakaryocytes co incubated with CRP and IL-6 or IL-6 alone expressed higher

Figure 3. Effects of interleukin-6 (IL-6) and C-reactive protein (CRP) on TNFRSF1B protein levels in human platelets. Platelets were treated with increasing concentrations of IL-6 or CRP or 0.1 U/mL of thrombin for 1 hour. A, Protein levels of TNFRSF1B were measured by Western blot from a representative healthy donor. B, Densitometry was performed on the represented Western blot. Actin served as a loading control. Values were compared with control (resting with and without buffer). C, Confirmation of the Western blot was performed by staining isolated, intact platelets with CD42b-fluorescein isothiocyanate (a platelet marker) and TNFRSF1B-PE antibodies. Isotype controls are shown in Figure 1D in the online-only Data Supplement. Representative photographs are shown. D, Fluorescence was determined using ImageJ (n=3), compared with control (with and without buffer).
levels of SIRPA than megakaryocytes not exposed to these inflammatory cytokines; however, this increase was not significant. Although the biological significance of these findings is unclear, our findings suggest a possible interaction between level of adiposity and SIRPA expression in platelets.

Our findings raise questions including causality and directionality. Although the cell culture experiment suggests alteration in gene expression consistent with the observed associations, it remains possible that levels of circulating cytokines are attributable to platelet gene expression or other unmeasured factor with residual confounding. Furthermore, our results do not completely answer the question as to whether obesity or inflammatory changes alter organ-based protein release and whether these inflammatory modulators alter gene expression in megakaryocytes and platelets (Figure 4). Although the studies are small, there are clinical observations that support this paradigm. Weight loss in 34 individuals led to decreased inflammatory cytokine gene expression including IL-6,51 and another had similar findings with severe calorie restriction.52

Although there have been other studies examining transcripts in blood, this study is unique in several respects. It reports individual genes from isolated cells using the more robust quantitative platform of quantitative reverse transcriptase polymerase chain reaction and, unlike microarray, does not use an unbiased approach. Although we have proposed some mechanisms for the observed effects, the cross-sectional nature of this analysis impairs our ability to discern the directionality of the association between circulating biomarkers and gene transcripts. In addition, the present study was conducted in middle-aged to elderly individuals of European descent; the generalizability to younger individuals or those of other ethnicity/race is unknown. In addition, our data will need to be replicated in an external cohort. Another limitation of this study is that platelet function testing, although performed during a prior FHS Offspring examination, was not performed during the eighth visit, thus preventing us from relating thrombotic markers, circulating inflammatory biomarker levels, and platelet gene expression.

Sources of Funding
This work was supported by N01-HC 25195, RFA-HL-12-008 (to J.E. Freedman, E. Mick), RO1 HL087201A (to J.E. Freedman, K. Tanriverdi), RFA-HL-12-008 (to J.E. Freedman), and 1RO1 HL64753, R01 HL076784, and 1 R01 AG028321 (to E.J. Benjamin) from the National Heart, Lung, and Blood Institute of the National Institutes of Health (NIH). Partial salary support is additionally provided by NIH grants 1U01HL105268-01 (to D.D. McManus) and KL2RR031981 (to D.D. McManus). L.M. Beaulieu is supported by P01 AI078894 from the National Institute of Allergy and Infectious Disease.

Disclosures
None.

References


Previous studies have examined genomic, transcriptomic, or circulating protein expression in large cohorts. There are significant difficulties in bridging protein and gene expression in these studies to gain insight into mechanistic or clinical questions. In the largest community-based study to date, we observed that 10 distinct platelet-derived inflammatory transcripts were associated with circulating C-reactive protein and interleukin 6 and that expression of several of these genes was influenced by level of obesity. Our cell culture experiment showed altered gene expression in megakaryocytes after exposure to C-reactive protein and interleukin 6, supporting our results and suggesting possible directionality for the associations observed using data from Framingham Heart Study Offspring participants. Our findings may provide insight into the transcriptomic mechanisms linking obesity, systemic circulating inflammatory mediators, and platelet inflammatory function in vivo. Further research is needed to determine the downstream effects of platelet mRNA expression on platelet function and atherogenesis.
Relationship Among Circulating Inflammatory Proteins, Platelet Gene Expression, and Cardiovascular Risk
David D. McManus, Lea M. Beaulieu, Eric Mick, Kahraman Tanriverdi, Martin G. Larson, John F. Keaney, Emelia J. Benjamin and Jane E. Freedman

Arterioscler Thromb Vasc Biol. published online August 22, 2013;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2013/08/22/ATVBAHA.112.301112

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/08/22/ATVBAHA.112.301112.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

Study Sample
The design and methods of the FHS Offspring study have been described.¹ The FHS Offspring Study is a prospective, community-based observational study of CVD and its risk factors. Study participants included children of the Original Framingham Heart Study cohort and their spouses.² Beginning in 1971, investigators enrolled 5,124 participants and evaluated these individuals approximately every 4 to 8 years. For our analysis, we focused on the attendees of examination cycle 8 (2005-2008) who underwent gene expression and biomarker measurement (n=1819). All study participants gave informed consent. The FHS protocol was approved by the Boston University Medical Center Review Board and University of Massachusetts Medical School Review Board.²

Risk Factor Assessment
As part of the FHS protocol, participants undergo medical history, laboratory assessment of risk factors, anthropometry, and targeted physical examination. Participants with systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mm Hg or receiving treatment for hypertension were defined as having hypertension. Body mass index (BMI) was calculated as the participant's weight in kilograms divided by the square of his/her height in meters (kg/m²). Diabetes was defined as having either a fasting plasma glucose ≥126 mg/dL or treatment with blood glucose-lowering medication.³ Participants were considered to be current smokers if they smoked ≥ 1 cigarette per day during the year prior to
examination. Criteria used for defining coronary heart disease and myocardial infarction in the FHS have been described elsewhere.2,4

**CRP and IL-6 Expression**

CRP and IL-6 were measured from venous blood samples obtained after participants completed an overnight fast. Fasting total cholesterol, HDL, and plasma glucose were assessed as previously described.4 High sensitivity CRP assays were performed using Dade-Behring nephelometer and reagents.5,6 IL-6 was assessed by enzyme-linked immunosorbent assay (R&D Systems HS600B).6 All intra-assay coefficients of variation were <9.2%. The assay range for CRP was 0.14-162.9 mg/L and 0.15 to 27.1 pg/ml for IL-6. After removing individuals with extreme elevations of CRP (>10 mg/L, N=106) and/or IL-6 (>10 pg/ml, N=112), the assay ranges in the remaining 1625 subjects was 0.14-9.9 mg/L for CRP and was 0.15 to 9.9 pg/ml for IL-6. The distribution of CRP and IL-6 assay levels in the restricted sample were not normally distributed and were consequently natural log (ln) transformed for statistical analysis.

Platelets were isolated from 35 cc of citrated venous blood through a series of centrifugation steps.7 White blood cell contaminants were removed using a 5 µm syringe filter. Through flow cytometry, confocal microscopy, and RNA assessment, leukocyte contamination of platelet samples was <1/50,000.7 Using an automated purification instrument, QIAcube (Qiagen) total RNA was then isolated from platelets using RNeasy Mini Kits (Qiagen). Following isolation, RNA concentration was measured using a NanoDrop ND-1000
spectrophotometer. RNA was converted to cDNA using cDNA reverse transcription kits (Applied Biosystems 4368813) and then pre-amplified using TaqMan PreAmp Master Mix (Applied Biosystems 4391128). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with a high throughput BioMark system (Fluidigm).

We examined platelet-derived RNA and not other cell types because we have shown that, of the circulating blood cells, platelet gene expression correlates significantly with CVD risk factors, specifically BMI and diabetes. We selected 15 genes all of which were known to be expressed in platelets and have an inflammatory function. Nine of the 15 genes signal through NFκB pathway. Twelve of the 15 were previously shown to have higher expression with greater mean BMI.

**Megakaryocyte Gene Expression**

A megakaryoblast cell line, Meg-01 cells (ATCC), was cultured in RPMI 1640 with L-glutamine, 1% antibiotic/antimycotic, and 10% heat-inactivated fetal bovine serum (Gibco). Cells were treated for 24 hours with vehicle, 20 µg/mL CRP (R&D Systems 1707-CR-200), 10 pg/mL IL-6 (R&D Systems 206-IL), or 20 µg/mL CRP and 10 pg/mL IL-6. After 24 h, total RNA was isolated using miRNeasy kits (Qiagen). RNA was converted to cDNA and pre-amplified using High-capacity cDNA Reverse Transcription Kit and TaqMan® PreAmp Master Mix (Applied Biosystems 4368813 and 4391128, respectively), respectively. Real-Time PCR was performed on an Applied Biosystems 7900 HT Fast Real-
Time PCR System with SDS 2.2.2 software using primers and probes for 15 specific genes and TaqMan® Gene Expression Master Mix (Applied Biosystems 4369016).

C57BL/6J mice (wildtype; WT) from the Jackson Laboratory (000664) were housed and maintained at the UMMS School Animal Facility. All animal experiments were approved by the University's Institutional Animal Care and Usage Committee. Bone marrow was isolated and cultured as previously described. On day 4, cultures were treated with increasing concentrations of rmIL6 or rmCRP (both from R&D Systems 406-ML-025 and 1829-CR-200, respectively) or both for 24 hours. Mouse megakaryocytes were isolated through magnetic bead separation (Miltenyi Biotec 130-097-146) using established methods and lysed using Qiazol (Qiagen). RNA was isolated and analyzed using the miRNeasy kit.

**Platelet mRNA Experiments**

Human whole blood from healthy donors was acquired using a protocol approved by the UMMS Institutional Review Board. Platelets were isolated and incubated with increasing concentrations of hrIL6 or hrCRP (R&D Systems) or human α-thrombin (Enzyme Research Labs) for 1 hour at 37°C. Platelets were lysed using Qiazol and RNA was isolated as described above.

**Immunofluorescence**

Mouse bone marrow or human platelets were fixed using 4% paraformaldehyde after treatment. Permeabilized mouse bone marrow was stained using anti-
CD41-FITC antibodies (BD Bioscience 553848) and anti-NFκBIA antibodies and a Texas Red secondary antibody (Abcam ab7217 and ab27478, respectively). Platelets were stained with anti-CD42b-FITC antibodies (eBioscience 11-0429) and anti-TNFRSF1b-PE antibodies (BD Bioscience 552418). Images were visualized using a Nikon TE-2000E2 inverted microscope with Solamere Technology Group modified Yokogawa CSU10 Spinning Disk Confocal Scan Head with Metamorph Software in the Digital Microscopy Core at the UMMS. Fluorescence measured using ImageJ v.1.46r (NIH). All photographs of isotype controls are shown in Supplemental Figures IA and D.

**Western Blots**

Western Blots were run and analyzed using ImageJ. All primary antibodies were purchased from Cell Signaling Technology (TNFRSF1b 3727) or Santa Cruz Biotechnology (Actin sc-1615 and CD45 sc-1123).

**Statistical Analyses**

All statistical analyses were performed using STATA 12.0. Descriptive statistics are displayed as mean ± SD for continuous variables and count (percentage) for categorical variables. The qRT-PCR results cycle threshold (Ct) values were standardized using 3 reference genes [β actin (ACTB), β2-microglobulin (B2M), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], which were previously found to be highly correlated. Genes not expressed, i.e., failing to surpass the Ct within a set period, were assigned the maximum Ct value (30
cycles) allowed according to our qRT-PCR procedures. Multivariable linear regression models tested for association between and gene expression (dependent variables) in platelets and inflammatory biomarker levels (ln transformed, exposure variables) adjusted for the following potential confounding variables (all assessed at the same examination RNA was collected): BMI, smoking status, total to HDL cholesterol ratio, triglycerides, systolic blood pressure, diastolic blood pressure, glucose level, diabetes, prevalent coronary heart disease, lipid-lowering therapy, hormone replacement therapy, antihypertensive therapy, and regular aspirin use (at least 3× per week). Since platelet gene profiles may differ between obese and non-obese individuals (BMI ≤ 30 kg/m²), we assessed the interaction of obesity status and gene ΔCt levels for each platelet-derived gene and inflammatory biomarker. To account for the number of statistical comparisons conducted for each gene and biomarker combination (that is 30 regression models) we employed false discovery rate (FDR) correction set at 5%.

*In vitro* differences in gene expression of megakaryocytes in response to CRP and IL-6 were assessed with regression models using the vehicle and non-treatment samples as the control group for reference. We considered p < 0.05 statistically significant for this experimental data presented.

References


