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Host factors associated with serologic inflammatory markers assessed using multiplex assays

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Abstract

Chronic systemic inflammation contributes to the development of adverse health conditions, yet the influence of fixed and modifiable risk factors on many serologic biomarkers of inflammation remains largely unknown. Serum concentrations of twenty-three biomarkers, including C-reactive protein (CRP), cytokines (CXCL11, CXCL8, CXCL10, CCL2, CCL13, CCL4, CCL17, CXCL13, IL-10, IL-12p70, IL-6, TNF-α, IL-2, IFN-γ, IL-1β, GM-CSF, BAFF), and soluble immune receptors (sCD14, sIL-2Rα, sCD27, sgp130, sTNF-R2) were measured longitudinally using multiplexed immunometric assays in 250 HIV-uninfected men followed in the Multicenter AIDS Cohort Study (1984–2009). Generalized gamma regression was used to determine the statistical significance of factors associated with each biomarker. After accounting for age, race, and education, and for analysis of multiple biomarkers, higher concentrations of specific individual biomarkers were significantly (P<0.002) associated with hypertension, obesity, hepatitis C infection, stimulant use, and diabetes and lower concentrations with hypercholesterolemia. These associations should be taken into account in epidemiological studies of these biomarkers, and may provide potential targets for disease prevention and treatment.

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Keywords

immune activation; cytokines; chemokines; cytokine receptors; C-reactive protein; risk factors

1. Introduction

Chronic inflammation and immune activation, as measured by concentrations of circulating inflammatory biomarkers, such as cytokines and chemokines, are associated with increased risk of several chronic diseases, such as cancer, cardiovascular disease (CVD), diabetes, AIDS, kidney disease, and aging.[1–8] Elevated levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, and acute phase proteins such as C-reactive protein (CRP), are prognostic of CVD outcomes, including acute myocardial infarction, congestive heart failure, and death, with CRP now being recommended in global risk prediction models for CVD.[9–11] Inflammation and dysregulated immune activation also have an etiologic role in carcinogenesis,[12,13] for example, in hepatocellular carcinoma due to infection with hepatitis B or C virus.[14] Elevated levels of B-cell stimulating cytokines, including IL-4 and IL-6, have been associated with AIDS-related non-Hodgkin lymphoma, possibly due to cytokine-mediated hyperstimulation of B-cell proliferation.[2,15–17] Inflammation may also contribute to obesity-related insulin resistance and by promoting macrophage infiltration into pancreatic islets.[18] Thus, understanding the host characteristics that influence inflammation is critical for understanding etiologic pathways, potential targets for preventive and therapeutic interventions, and risk stratification, and for the design of valid epidemiologic studies to define these.

Inflammatory pathways are complex and often involve overlapping immune processes. However, most studies have analyzed small numbers of inflammatory biomarkers, such as C-reactive protein (CRP), an acute phase reactant, and the pro-inflammatory cytokines IL-6 and TNF-α. This has greatly limited understanding of the relationships between inflammation and sociodemographics, risk behaviors, and morbidities. However, now that multiplex assay technologies are available that permit much more extensive characterizations using small quantities of serum,[19] this situation is starting to improve. For example, a recent study of healthy women found that TNF-α, IL-1β, IL-2, sIL-2Rα, IL-10, and IL-12p40/70 were significantly associated with age, body mass index and reproductive factors.[20]

With the foregoing in mind, the objective of this study was to evaluate the association of sociodemographics, risk behaviors, and select morbidities, hypothesized *a priori*, with circulating concentrations of 23 markers including cytokines, chemokines, soluble immune receptors, and CRP. In addition to the morbidities noted above, obesity was analyzed because, as mentioned, it has been associated with inflammation.[21,22] Behaviors analyzed included smoking, alcohol consumption, and use of recreational substances, including marijuana, amyl nitrates, and stimulants, that may stimulate the inflammatory system.[20,23–32] For these analyses, we capitalized on the existence of specimens collected and stored with standardized methods in a long-term longitudinal study with well-characterized participants.

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2. Materials and Methods

2.1. Study population and design

The Multicenter AIDS Cohort Study (MACS) is an ongoing prospective study of HIV-infected and HIV-uninfected men who have sex with men enrolled in Baltimore/Washington D.C., Chicago, Los Angeles, and Pittsburgh; 6,972 men were enrolled from 1984 through 2003. Standardized interviews and physical examinations were administered at semiannual study visits, including specimen collection for storage and testing in a national repository. A full description of study procedures has been published.[33] Study documentation may be found at http://www.statepi.jhsph.edu/macs/macs.html. The institutional review board at each center approved the MACS protocols; informed consent was obtained from all participants.

We capitalized on the availability of archived serum obtained from the study visit to examine the effects of host factors on these biomarkers. Serum samples were processed within 6 hours of blood draw and stored at −80°C. Prior to testing, a previously unthawed stock vial was thawed for each study visit, aliquoted, and stored at −80°C until testing. The study was restricted to HIV-uninfected men to exclude the effect of HIV infection on these markers. We aimed to sample 4 longitudinal visits approximately 5 years apart for each of 250 HIV-uninfected participants, spanning the duration of the MACS (1984–2009 at the time); 90% (n=224) of these participants had samples available at all 4 visits, 9% (n=23) had 3 samples and the other 3 people had 1–2 samples. These men were randomly selected from 1,012 persistently HIV-uninfected men in the MACS with ≥4 longitudinal visits, with the exception that all HIV-uninfected men who had chronic hepatitis C infection were selected to obtain sufficient numbers of HCV-infected men to examine the effect of this infection on the markers studied.

2.2. Laboratory methods

Two electrochemiluminesence-based multiplex assay panels (Proinflammatory 9-plex and Chemokine 7-plex; Meso-Scale Diagnostics, LLC, Rockville, MD) were used to determine concentrations of IL-1β, IL-2, IL-6, IL-10, IL-12p70, GM-CSF, IFN-γ, TNF-α, CXCL8 (IL-8), CXCL10 (IP-10), CCL11 (eotaxin), CCL2 (MCP-1), CCL13 (MCP-4), CCL4 (MIP-1β), and CCL17 (TARC). All testing was done at a centralized laboratory. Analyte- and plate-specific lower limits of detection (LLOD) were calculated as concentrations 2.5 standard deviations above the background for each analyte on each plate. Concentrations of five soluble receptors (sCD14, sgp130, sIL-2Rα, sTNF-R2), a cytokine (BAFF), and a chemokine CXCL13 (BLC-BCA1), were measured in a single panel (Human Biomarker Custom Premix Kit A) using the fluorescent bead-based multiplexed Luminex xMAP system at a centralized laboratory (Fluorokine® MAP, R&D Systems, Minneapolis, MN), and a Bio-Plex 200 Luminex instrument and Bio-Plex software (Bio-Rad, Hercules, CA). A single assay lot was used. Finally, CRP was measured at Quest Diagnostics using a high-sensitivity nephelometric assay (Dade Behring, Inc., Newark, DE). All specimens for any given individual were run on the same plate.

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2.3. Exposure variables

Sociodemographics included age at visit, race (non-black vs. black), and baseline educational level (four-year college degree or higher vs. less than college degree). Body-mass index (BMI = weight (kg)/height $(m)^2$) was categorized into clinically meaningful categories: ≤24.9 (normal/underweight), 25–29.9 (overweight), and ≥30 (obese). Data from the visit closest to the blood draw (≤1.5 years) were used to define time-varying exposures. Detailed behavioral data were collected from participants at each visit using interviewer-administered and/or audio computer assisted self-interview (ACASI) format. Behavioral factors included any sexual activity, risky sex (≥2 partners [male or female]), smoking status (never, former, current), alcohol consumption (binge-heavy drinking [≥5 drinks/day for ≥1 month], moderate-heavy [3–4 drinks/day for >1 month, or ≥5 drinks/day per month], low-moderate [1–2 drinks/day or 3–4 drinks/day for ≤1 month], or none), and use of recreational drugs (marijuana, amyl nitrates, or any stimulant [cocaine, ecstasy, methamphetamines, or any other uppers]). Hepatitis C infection (HCV) was categorized as negative, cleared [antibody+ only], or chronic [RNA positive]. Sexually transmitted disease was present if any new diagnosis of herpes virus infection, syphilis, genital warts, or gonorrhea was reported. Presence of depressive symptoms was defined as ≥16 on the Center for Epidemiologic Studies Depression Scale (CES-D).[34] An expanded categorization of depressive symptoms additionally included men who reported using medication for depression since the last visit, regardless of their CES-D score. Persistent diabetes (fasting glucose>126 mg/dl, hemoglobin A1c (HbA1c) ≥6.5%, or diabetic medication use) and persistent hypertension (systolic blood pressure (SBP) ≥140 mmHg, diastolic (DBP) ≥90 mmHg, or antihypertensive medication use) were defined if present at ≥2 visits prior to blood draw. Hypercholesterolemia was defined as fasting total serum cholesterol ≥200 mg/dl. Time of blood draw was P.M. versus A.M.

2.4. Statistical methods

Multivariate generalized gamma regression models were used to assess independent associations of exposures with individual biomarkers. The generalized gamma distribution is a flexible three-parameter distribution and permitted us to avoid making strong assumptions regarding the distributions of different biomarkers.[35] Biomarker concentrations were inversed so that measurements below the lower limit of detection could be handled as right-censored. In all models, only the location (β) parameter was allowed to vary by exposure category, while scale (σ) and shape (λ) were held constant. Relative percentiles (the exponential of the $-\beta$, to account for using the inverse value) in biomarker concentrations were calculated for each covariate category. Because σ and λ were held constant, the relative percentile comparing one exposure category to another is constant across all percentiles of each biomarker. Using the relative percentiles, we calculated the percent difference in biomarker concentrations between the exposed and unexposed [(relative percentile–1) × 100]. Robust standard errors adjusted for repeated measurements by using the vce(cluster) option in Stata. We also conducted logistic regression analyses accounting for correlated outcomes to determine associations between the factors and detectability of those cytokines with < 80% detectable values. All statistical tests were 2-sided. To account for multiple testing, a Bonferroni adjustment at an α-level of 0.05 [(0.05/23)=0.002] for

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statistical significance was employed. Results with $0.002 < P \leq 0.05$ were considered marginally significant.

Separate analyses were conducted for each biomarker, controlling first for age, study site, race, education, and time of blood draw. Subsequent models included modifiable factors: BMI, HCV, risk behaviors, and depressive symptoms. Because consistent ascertainment of morbidities (diabetes, hypertension, and hypercholesterolemia) began only in 2001, models examining these morbidities were restricted to data obtained from the period 2001–2009. For all models, age was centered at the median (46 years) across all 971 person-visits and associations per 10-years were assessed. The estimated differences are presented graphically, in the form of a heatmap, which permits examination of the associations of individual exposures with each biomarker as well as examining the biomarkers that are affected by the exposures. Color densities for the heatmap were standardized using the sample standard deviation of each exposure.

Analyses were conducted using SAS, version 9.3 (SAS Institute, Inc., Cary, North Carolina) and Stata, version 13 (College Station, Texas).

3. Results

The characteristics of the study population are described in Table 1. Of the 250 men, 52% (n=129) entered into the cohort before 2001, and 121 (48%) in 2001–3. Overall, the median time from first to last sample analyzed for an individual was 18.3 years (IQR: 5.5–19.8); the median interval between samples was 4.5 years (IQR: 2.6–12.8). The median age across all person-visits was 45.6 years (IQR: 39.7–52.5), and 55% of men were overweight or obese. Thirty percent reported having depressive symptoms, and 37% were classified as depressed when those using antidepressant medication were added to those with only depressive symptoms. Approximately 23% of the study group had chronic HCV infection, which reflects the original design of the larger cohort from which this group was drawn.

Table 2 shows the detectability and distributions of each biomarker across all person-visits. The chemokines, soluble receptors, IL-6, TNF-α, and BAFF were detected in all, or nearly all, samples. In contrast, GM-CSF, IL-2, IL-1β, and IFN-γ were detectable in <80% of samples.

Associations between each host factor and each biomarker, adjusting only for age, MACS study center, and time of day of blood draw are provided in Figure 1 in McKay.[36] Of the exposures studied, all were associated with at least one biomarker, with the exception of sexually transmitted disease and risky sex. Therefore, the multivariable models only included age, race, education, time of blood draw, BMI, HCV, smoking status, alcohol consumption, recreational drug use, and depressive symptoms using the entire sample (*i.e.*, 1984–2009) [Model 1], and also diabetes, hypertension and hypercholesterolemia using the restricted sample (*i.e.*, 2001–2009) [Model 2]. In these models, which are summarized in Figure 1, 16 of the 23 biomarkers were significantly associated with at least one of the examined host characteristics at the $P<0.002$ level, and the remaining 7 markers had associations at the $P<0.05$ level. The estimates in Figure 1 reflect percent differences in

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biomarker concentrations between the exposed and unexposed for each covariate presented. For example, chronic HCV was associated with 54% higher concentrations of IL-10 ($P<0.002$) (i.e., the overall distribution of IL-10 was 54% higher in those with chronic HCV vs. those who were HCV negative, controlling for age, race, education, time of blood draw, BMI, smoking status, alcohol consumption, recreational drug use, and depressive symptoms) and 54% lower concentrations of C-reactive protein (CRP) ($P<0.002$). The directionality of differences in detectability for the four cytokines with < 80% detectable values were similar to the results obtained from generalized gamma regression models.

3.1. Relationships of biomarkers with fixed host characteristics

Several of the biomarkers were influenced by fixed host characteristics. Older age was significantly ($P<0.002$) associated with higher concentrations of chemokines, cytokines and soluble receptors, specifically CXCL10, CCL13, IL-6, sCD27, and sTNF-R2. Race primarily influenced chemokine levels, in that being non-black was associated significantly with higher levels of CCL11 and CCL2 and lower levels of CCL13, CCL4, and CCL17, and marginally ($0.002<P\leq0.05$) with higher levels of TNF-α, BAFF, sCD14 and sIL-2Rα. IL-6 was the only biomarker associated with higher education. Compared to morning blood draws, specimens drawn in the afternoon/evening had significantly lower concentrations of IL-2; their lower CXCL10 and higher CXCL13 levels were of marginal significance.

3.2. Relationships of biomarkers with modifiable host characteristics

A number of modifiable characteristics were strongly associated with several of the biomarkers. Men with chronic HCV infection had significantly ($P<0.002$) higher concentrations of CXCL10, IL-10, BAFF, sIL-2Rα, sCD27, sgp130, and sTNF-R2, and lower concentrations of CRP than uninfected men, and marginally significantly lower levels of CCL11, CCL13, and CCL17. Men with cleared HCV infection had significantly higher concentrations of IL-6 than both uninfected men and men with chronic HCV infection.

Individuals who were overweight or obese had significantly higher IL-6 and CRP. Obesity was further associated with significantly higher levels of CXCL10 and lower levels of CXCL13, and marginally with other biomarkers.

Associations between behaviors and biomarkers were mostly of marginal significance ($0.002<P\leq0.05$). The only significant association was between recreational stimulant use and higher levels of IL-10. Marijuana use was marginally associated with lower concentrations of TNF-α, sCD27, sgp130, and sTNF-R2, while the use of amyl nitrates was associated with higher concentrations of CXCL10 and lower concentrations of IL-12p70. Compared to non-smokers, current smokers had lower concentrations of CXCL10, and higher concentrations of BAFF and CRP, with the latter association approaching statistical significance ($P = 0.003$). Compared to non-drinkers, those who reported moderate-heavy drinking had lower CCL17, IL-6, TNF-α, and sTNF-R2 and higher concentrations IL-12p70 and sCD14. High values of IL-12p70 and sCD14 were also associated with binge drinking.

Men with depressive symptoms defined by CES-D>16 had higher CXCL10 and sCD27, but these associations were of marginal significance. The addition of men taking antidepressants to the group with depressive symptoms attenuated the association with sCD27 and revealed

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marginally significant associations of depression with higher values of CXCL8, IL-6, and TNF-α.

3.3. Relationships of biomarkers with morbidities

The last three columns of Figure 1 show the results from Model 2, which included, in addition to the factors in Model 1, three morbidities: persistent diabetes, persistent hypertension, and hypercholesterolemia. This analysis included only data from 2001–09. The associations found in Model 1 were essentially unchanged (see Figure 2 in McKay[36]), except that current smoking was associated with higher CXCL8 (PD = 23; $P = 0.01$) and IL-6 and CXCL10 were no longer associated with age after accounting for these morbid conditions. Men with persistent diabetes had significantly higher concentrations of IL-6 than those without diabetes; IL-2, IFN-γ, sCD14, and sCD27 levels were marginally significantly higher. These associations did not change when restricting the analysis to those with uncontrolled diabetes. Concentrations of CXCL8 were significantly higher in the presence of controlled or uncontrolled hypertension. However, IL-6 levels were only significantly higher among those with uncontrolled hypertension compared to normotensive persons. Levels of CXCL13 and IL-10 were lower in those with uncontrolled hypertension. Finally, hypercholesterolemia was associated with significantly lower concentrations of CXCL13 and sCD27, marginally significantly lower levels of sIL-2Rα and sTNF-R2, and higher levels of CCL13 and CRP. These associations were mostly due to changes in HDL versus LDL (Table 3).

4. Discussion

Our results demonstrate that the inflammatory biomarkers investigated here were affected by sociodemographic and behavioral risk factors and by select morbidities. These findings are highly relevant for researchers investigating the role of these biomarkers in disease pathogenesis. Further, identifying modifiable risk factors that are associated with changes in these biomarkers may facilitate the development of clinical and behavioral interventions for inflammation-associated conditions. Finally, the observed associations between biomarkers and fixed characteristics indicate variables that need to be considered and controlled for when examining these biomarkers in epidemiological studies. To our knowledge, this is one of the largest studies to date to examine the relationships between host characteristics on a broad panel of inflammatory biomarkers.

The factor that affected these biomarkers the most was chronic HCV infection. Chronic HCV infection is a major risk factor for hepatocellular carcinoma (HCC), with evidence suggesting that HCV-induced inflammatory pathways are the primary mechanisms through which hepatocellular carcinogenesis is initiated.[37] The identification of novel biomarkers associated with HCV may aid in the development of therapeutic targets for modifying inflammatory mediators. In our study, HCV infection was associated with higher CXCL10, IL-10, BAFF, sIL-2Rα, sCD27, sgp130, and sTNF-R2 and depressed CRP. The biomarkers that were higher in men with chronic HCV are consistent with the activation of immune cells by HCV. CXCL10, which is secreted by hepatocytes and is a chemoattractant for monocytes/macrophages, natural killer cells, T cells, and dendritic cells,[38,39] has been used as a marker

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of HCV treatment outcome, with higher pre-treatment concentrations associated with greater risk of non-response.[40] Our observation that chronic HCV was associated with CXCL10 levels that were 143 percent higher than those without HCV infection is consistent with the known effects of HCV on CXCL10.[40] In the present study, chronic HCV was also associated with higher levels of the immune activation markers sTNF-R2, sIL-2Rα, and sCD27. This degree of difference in sIL-2Rα approaches that associated with a change in the International Prognostic Index risk group from Good to Very Good for patients with diffuse large B-cell lymphoma.[41] Similarly, the percent difference in sCD27 associated with chronic HCV in this study approaches the levels that distinguished between non-Hodgkin lymphoma cases and controls observed in an earlier MACS study.[42] These findings together with others support a role for inflammation in carcinogenesis.[14,43,44] Another study recently showed that sTNF-R2 levels were associated with prognosis among those with chronic kidney disease, whereby the percent difference in baseline median values was smaller than the differences by HCV infection in the present study.[45] sTNF-R2 and sIL-2Rα play a role in T cell activation, growth, and proliferation,[46,47] and sCD27 interacts with IL-2 to promote CD8+ T cell effector function.[48] sIL-2Rα, an essential component of IL-2 signaling, has been used as a measure of T cell activation and a marker of memory B cells.[49] BAFF, expressed by macrophages, dendritic cells, and neutrophils, is a potent B cell activator.[50] Interestingly, IL-6 was significantly associated with cleared HCV infection, but not chronic (*i.e.*, active) HCV infection. This finding could be explained by the fact that sgp130, which is elevated in chronic HCV infection, is an inhibitor of IL-6;[51] thus, it could be that normalization of sgp130 levels after HCV clearance unmasks elevations in IL-6. The decreased CRP levels in HCV chronically-infected persons has also been shown by others,[52,53] providing external validity to these results.

Obesity was also strongly associated with biomarkers of inflammation, specifically with higher IL-6, CRP, CXCL10 and lower CXCL13. Obesity has been implicated in the development of a host of chronic conditions, including cardiovascular disease, diabetes, and several cancers, primarily through inflammatory-mediated pathways originating from adipose tissue.[54] Adipose tissue produces large amounts of inflammatory molecules, including IL-6 and CXCL10.[21] In the present study, levels of both IL-6 and CXCL10 were nearly 30% higher in obese men than in those with normal BMI.[21] These findings are consistent with a recent report of higher IL-6, CRP and TNF-α associated with increasing BMI among women.[20] The size of this effect on IL-6 is clinically meaningful; for example, in one study, increases in IL-6 levels of 40% were associated with 38% elevated risk of myocardial infarction in otherwise healthy men.[55] An association between obesity and lower levels of CXCL13, a B-cell chemoattractant, has also been reported in women.[56]

Risk behaviors also affected specific biomarkers. For example, CRP levels were 53% higher in current smokers than in nonsmokers, consistent with a proinflammatory role of smoking in carcinogenesis and atherogenesis. Current smoking was also associated with lower CXCL10 concentrations, suggesting that smoking may suppress anti-tumor activity by effector T-cells. Otherwise, smoking was not significantly associated with the other biomarkers studied. Reports vary widely on the effect of smoking on inflammatory biomarkers.[20,23,24,57] This variation may be due to heterogeneous populations, residual confounding (*e.g.*, the cumulative effects of exposure using units of pack-years vs. using the

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current categorizations), and not controlling for BMI which, as discussed above, is correlated with concentrations of circulating pro-inflammatory biomarkers.

Regarding the immunomodulatory effects of recreational drug use, marijuana use was associated with lower sCD27, sTNF-R2, sgp130, and TNF-α concentrations, although the effect sizes were all <10%. Nevertheless, this is consistent with a growing body of research that suggests cannabinoids inhibit the co-stimulatory activity of macrophages and suppress the release of pro-inflammatory mediators.[58,59] The therapeutic potential of cannabinoids as anti-inflammatory agents has been examined in clinical studies of multiple sclerosis, arthritis, and traumatic brain injury.[28] Further evaluation of the putative anti-inflammatory effects of cannabinoids should be examined to characterize their therapeutic utility. Interestingly, stimulant use was associated with significantly elevated concentrations of IL-10, an anti-inflammatory, B cell-stimulatory cytokine secreted by T-regulatory cells, suggesting an immunosuppressive response. The increased IL-10 in stimulant users provides additional support for a pathway between use of stimulants and increased NHL risk, as reported previously.[16,60–62] In a recent meta-analysis, higher IL-10 was associated with adverse survival in many types of cancer, suggesting the potential clinical utility of IL-10 antagonists in cancer treatment.[63] We did not observe an elevation in pro-inflammatory cytokines or CRP with stimulant use, as described by others.[32,64] This discrepancy could be due to our summarization of any stimulant use, which may have masked heterogeneity associated with different drugs. Alternatively, a threshold may exist below which the immune system is not significantly influenced.

Although these participants were generally free of morbidity, observed associations were consonant with known relationships. The inverse association between hypercholesterolemia with sIL-2Rα, sCD27 and sTNF-R2 was likely due to the anti-inflammatory nature of HDL.[65] CRP was the only biomarker directly associated with LDL, consistent with our knowledge of atherogenesis.[66] Although we observed a strong association of diabetes with the pro-inflammatory marker IL-6, as shown by others,[4,67,68] we did not find a significant association with TNF-α or CRP. One possible explanation is the low prevalence of diabetes in our study population, thus limiting our power to detect associations with small effects. Concentrations of CRP were generally low in our population, thus it is likely that these men were at lower risk for many morbidities. The association of uncontrolled hypertension with significantly higher CXCL8 and IL-6 is consistent with evidence implicating T cell cytokines in vascular disease.[69] Interestingly, when adjusting for these co-morbidities, most of the associations between markers of immune activation with age persisted, but the associations with inflammatory markers IL-6, CXCL8, and CXCL10 attenuated and became non-significant. This suggests that that the excess inflammatory burden observed with age may be explained by age-related morbidities. However, other pathways leading to immune activation with increasing age should be considered.

Depressive symptoms, as defined by a CES-D score >16, was associated with higher levels of CXCL8, CXCL10, IL-6, and TNF-α when we included men who reported use of antidepressant medications, possibly because use of these medications may reflect clinically-defined depression and improve the classification. These findings are consistent with those published by others.[70,71] While depression may promote a dysregulated immune response

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through multiple pathways (*e.g.*, adiposity),[72,73] it is also proposed to have an inflammatory pathogenesis. Additional prospective examination of the relationship between inflammation and depression is needed to establish the temporal relationship. It is a limitation of this study that the medication class and the adequacy of disease control were both unknown. Further research might clarify biomarker-mediated pathways.

As noted above, increasing evidence suggests that differences in inflammatory biomarkers are clinically informative. CRP has perhaps been the most frequently investigated, with elevated levels associated with 2-fold increased risk of cardiovascular disease even after controlling for traditional cardiovascular risk factors such as age, family history, and smoking.[74] A doubling of CRP levels has also been implicated in increased risk of hepatocellular carcinoma[75] and cognitive impairment.[76] In the present study, the median CRP level for men with normal BMI was 0.1 mg/L and was more than doubled for men with overweight and obese BMI (1.1 mg/L, and 2.2 mg/L, respectively). Current smoking and hypercholesterolemia were also associated with elevated CRP levels (PD = 53% and 28%, respectively).

Emerging evidence also suggests that inflammatory biomarker levels are associated with risk of certain cancers in addition to non-Hodgkin lymphoma. For example, a doubling of IL-6 has been associated with an increased risk of epithelial ovarian cancer, hepatocellular carcinoma, and lung cancer.[1,57,75,77] In the present study, differences of 15–34% in levels of IL-6 were associated with increasing BMI, cleared HCV, and persistent diabetes, while smaller increases were observed with increasing age and depression. It also is important to consider that relatively small effect sizes may contribute to risk in a synergistic manner. Determination of the clinical meaningfulness of these inflammatory biomarkers will require standardized laboratory measurements and additional studies that consistently find that the same cutoffs predict disease, as was done for CRP.

The study had some limitations. Biomarkers could have degraded in storage of the specimens studied. However, non-differential degradation will bias toward the null and differential degradation by exposure is unlikely given the random selection of person-visits studied. Thus, the estimates presented are likely to be conservative. Further, restricting the analysis to samples collected from 2001–2009 did not change the results, which also argues against a problem with early specimens. Some potentially important mediators or confounders, *e.g.*, use of anti-inflammatory medications and physical activity, could not be studied. In addition, the possibility of acute illness at the time of blood draw was not accounted for in our analysis. However, we would not expect that the prevalence of such acute illnesses would differ by the exposure categories examined here. This non-differential effect would bias results toward the null; therefore, the results of this study would be conservative. Although generalizability to other populations, such as women or children, may not be appropriate, studies performed in women have shown similar associations with shared biomarkers.[20,27] Because reference ranges have not been established for most of the studied biomarkers, inferences from this study must be limited to relative distributions, rather than absolute cutoffs. Finally, our study involved a considerable number of statistical comparisons. Although adjustment for multiple comparisons was conducted using a Bonferroni correction, which is considered conservative, it remains possible that some

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associations were significant solely due to chance. The observed associations should therefore be interpreted with caution and be further evaluated in independent populations.

This study also has several strengths. The MACS is a large, longstanding prospective cohort study of men followed for more than 25 years with in-depth standardized data collection and specimen collection, processing and storage. This permitted the evaluation of diverse correlates of immune activation with a precision generally not possible in smaller studies. Person-visits were selected to reflect the entire period of cohort follow-up (1984–2009) and to contain important subgroups (*e.g.*, African-Americans and those with chronic HCV). Multiplex testing allowed for simultaneous quantitation of a broad spectrum of biomarkers using small sample volumes, reducing cost and improving the efficient use of valuable stored specimens. Testing of previously unthawed specimens was conducted in single laboratories for given analytes, with all longitudinal specimens per individual run on the same plate to minimize assay variability. Finally, innovative statistical methods, *i.e.*, generalized gamma regression, were utilized to incorporate observations below limits of detection. Other methods, *e.g.*, assigning undetectable levels with ½ LLOD, may result in estimates with overly narrow confidence intervals, yielding incorrect inferences. Adjustment for multiple testing was used to reduce the likelihood of false-positive findings. Finally, data on risk behaviors, including self-reported drug use, have been captured through an ACASI software system, a standardized method for data collection that safeguards privacy and has been shown in numerous populations to increase the accurate and unbiased reporting of behaviors that may be socially sensitive.[78–80]

Our study has both analytic and clinical implications. Fixed (*e.g.*, race) and modifiable (*e.g.*, obesity, HCV infection) host characteristics suggest potential confounders for future etiologic studies of inflammation and potential targets for risk prediction, prevention, and therapeutic interventions. These findings will inform both our understanding of cellular interactions in the immune system and potential inflammatory pathways in chronic disease development.

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Abbreviations

ACASI	audio computer assisted self-interview
BAFF	B-cell activating factor

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BMI	body mass index
CRP	C-reactive protein
CI	confidence interval
CCL11	C-C motif ligand 11
CES-D	Center for Epidemiologic Studies Depression Scale
CXCL8	C-X-C motif ligand 8
CVD	cardiovascular disease
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDL	high-density lipoprotein
HIV	human immunodeficiency virus
IFN	interferon
IL	interleukin
sIL-2Rα	soluble IL-2 receptor alpha
LDL	low-density lipoprotein
MACS	Multicenter AIDS Cohort Study
NHL	non-Hodgkin lymphoma
sCD14	soluble cluster of differentiation 14
sgp130	soluble glycoprotein 130
TNF-α	tumor necrosis factor-alpha
sTNF-R2	soluble tumor necrosis factor-receptor 2

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Highlights

- Body mass index was associated with higher IL-6, CRP, and CXCL10 and lower CXCL13.
- Hepatitis C was associated with higher CXCL10, IL-10, BAFF, sIL-2Rα, sCD27, sgp130, sTNF-R2 and lower CRP.
- Levels of IL-10 were higher in stimulant users than non-users.
- Depressive symptoms were associated with higher CXCL10.
- Diabetes and hypertension were associated with higher IL-6 and CXCL8, respectively.

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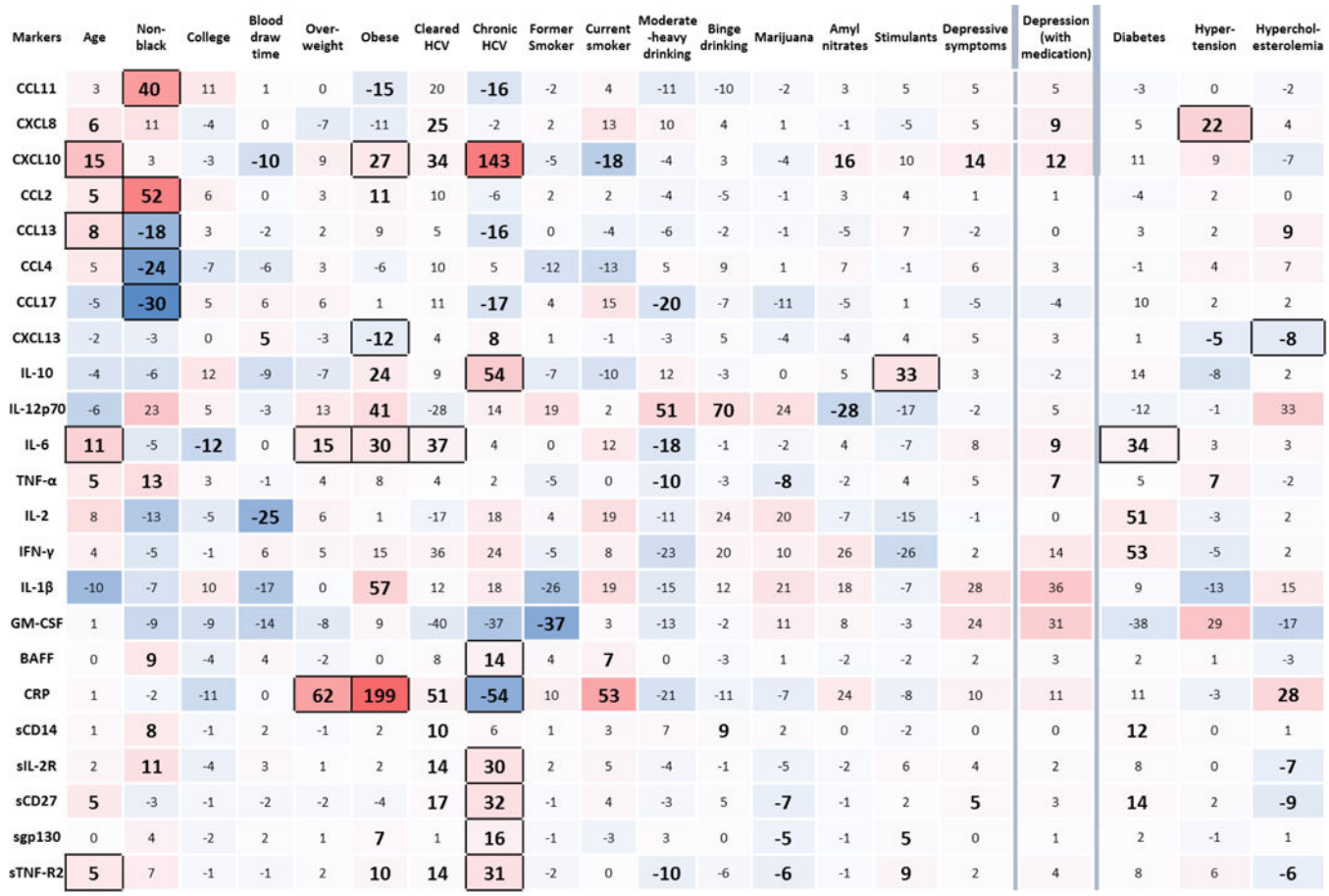

Markers	Age	Non-black	College	Blood draw time	Over-weight	Obese	Cleared HCV	Chronic HCV	Former Smoker	Current smoker	Moderate-heavy drinking	Binge drinking	Marijuana	Amyl nitrates	Stimulants	Depressive symptoms	Depression (with medication)	Diabetes	Hyper-tension	Hypercholesterolemia
CCL11	3	40	11	1	0	-15	20	-16	-2	4	-11	-10	-2	3	5	5	5	-3	0	-2
CXCL8	6	11	-4	0	-7	-11	25	-2	2	13	10	4	1	-1	-5	5	9	5	22	4
CXCL10	15	3	-3	-10	9	27	34	143	-5	-18	-4	3	-4	16	10	14	12	11	9	-7
CCL2	5	52	6	0	3	11	10	-6	2	2	-4	-5	-1	3	4	1	1	-4	2	0
CCL13	8	-18	3	-2	2	9	5	-16	0	-4	-6	-2	-1	-5	7	-2	0	3	2	9
CCL4	5	-24	-7	-6	3	-6	10	5	-12	-13	5	9	1	7	-1	6	3	-1	4	7
CCL17	-5	-30	5	6	6	1	11	-17	4	15	-20	-7	-11	-5	1	-5	-4	10	2	2
CXCL13	-2	-3	0	5	-3	-12	4	8	1	-1	-3	5	-4	-4	4	5	3	1	-5	-8
IL-10	-4	-6	12	-9	-7	24	9	54	-7	-10	12	-3	0	5	33	3	-2	14	-8	2
IL-12p70	-6	23	5	-3	13	41	-28	14	19	2	51	70	24	-28	-17	-2	5	-12	-1	33
IL-6	11	-5	-12	0	15	30	37	4	0	12	-18	-1	-2	4	-7	8	9	34	3	3
TNF-α	5	13	3	-1	4	8	4	2	-5	0	-10	-3	-8	-2	4	5	7	5	7	-2
IL-2	8	-13	-5	-25	6	1	-17	18	4	19	-11	24	20	-7	-15	-1	0	51	-3	2
IFN-γ	4	-5	-1	6	5	15	36	24	-5	8	-23	20	10	26	-26	2	14	53	-5	2
IL-1β	-10	-7	10	-17	0	57	12	18	-26	19	-15	12	21	18	-7	28	36	9	-13	15
GM-CSF	1	-9	-9	-14	-8	9	-40	-37	-37	3	-13	-2	11	8	-3	24	31	-38	29	-17
BAFF	0	9	-4	4	-2	0	8	14	4	7	0	-3	1	-2	-2	2	3	2	1	-3
CRP	1	-2	-11	0	62	199	51	-54	10	53	-21	-11	-7	24	-8	10	11	11	-3	28
sCD14	1	8	-1	2	-1	2	10	6	1	3	7	9	2	0	-2	0	0	12	0	1
sIL-2R	2	11	-4	3	1	2	14	30	2	5	-4	-1	-5	-2	6	4	2	8	0	-7
sCD27	5	-3	-1	-2	-2	-4	17	32	-1	4	-3	5	-7	-1	2	5	3	14	2	-9
sgp130	0	4	-2	2	1	7	1	16	-1	-3	3	0	-5	-1	5	0	1	2	-1	1
sTNF-R2	5	7	-1	-1	2	10	14	31	-2	0	-10	-6	-6	-1	9	2	4	8	6	-6

Figure 1.

Percent differences from multivariate generalized gamma regression models examining the associations of age, non-black race, college education at baseline, blood draw time (P.M. versus A.M.), being overweight, obesity, cleared (antibody positive only) hepatitis C infection (HCV), chronic hepatitis C infection, former smoking, current smoking, moderate-heavy alcohol consumption, binge alcohol consumption, use of marijuana, use of amyl nitrates, use of stimulants, the presence of depressive symptoms, and depression including those taking depression medication (*column headings*) with individual biomarkers (*row headings)*, using the full sample (1984–2009). Percent differences in biomarker concentrations associated with persistent diabetes, persistent hypertension, and hypercholesterolemia are adjusted for age, study site, race, baseline education, blood draw time, body mass index, depressive symptoms, hepatitis C infection (HCV), smoking status, alcohol consumption, and recreational drug use, using the restricted sample (2001–2009). Large **bold** text **with** black-bordered cells indicates significance at the $P<0.002$ level; large **bold** text **without** borders indicates marginal significance ($0.002<P\leq0.05$). The color gradient of each cell illustrates the magnitude of the percent difference in biomarker concentration (darker red indicating stronger positive percent difference and darker blue indicating stronger negative percent difference). For example, chronic HCV is associated

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with 54% higher concentrations of IL-10 ($P<0.002$) and 54% lower concentrations of C-reactive protein (CRP) ($P<0.002$).

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Table 1

Characteristics of person-visits from 250 HIV-uninfected men from the Multicenter AIDS Cohort Study (MACS), 1984 – 2009

Sociodemographics	Median (IQR) or %
	Overall (*N* = 971)
Age at blood sampling (years), median (IQR)	45.6 (39.7–52.5)
≤30	7
30.1 ≤ 40	19
40.1 ≤ 50	41
50.1 ≤ 60	24
60.1 ≤ 70	8
70.1+	1
Race	
Non-black	61
Black	39
Education (at baseline)	
Four-year college degree or higher	47
Less than college degree	53
Body mass index at blood sampling, kg/m^2	
Obese (≥30)	19
Overweight (25–29.9)	37
Normal/underweight (≤24.9)	45
Hepatitis C infection at blood sampling	
Chronic	23
Cleared (Antibody+ only)	7
Negative	70
CES-D Score, mean (SD)[a]	11
Depressive symptoms[b]	
Yes	29
No	72
Depression[c]	
Yes	37
No	63
Study site	
Baltimore/Washington D.C.	22
Chicago	24
Pittsburgh	35
Los Angeles	19
Time of blood draw	
A.M.	54
P.M.	47

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Behaviors	**Median (IQR) or % Overall (*N* = 971)**
Smoking status at blood sampling	
Current	41
Former	35
Never	24
Drinking classification at blood sampling[d]	
Binge	11
Moderate-heavy	20
Low-moderate	50
Non drinker	19
Marijuana use since last visit	
Yes	31
No	69
Amyl nitrates use since last visit	
Yes	19
No	81
Stimulant use since last visit[e]	
Yes	20
No	80
Risky sex since last visit[f]	
Yes	46
No	54
Sexually transmitted infection since last visit[g]	
Yes	14
No	86
Morbidities (2001 – 2009)	*N* = 720
Persistent diabetes mellitus[h]	
Yes	9
No	91
Uncontrolled diabetes[i]	
Yes	7
No	93
Persistent hypertension[j]	
Yes	49
No	51
Uncontrolled hypertension[k]	
Yes	30
No	70
Total cholesterol, mg/dl[l]	
Borderline-high (≥200)	34
Desirable (<200)	66

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Behaviors	Median (IQR) or % Overall (*N* = 971)
High density lipoprotein (mg/dl)	49 (40 – 58)
Low density lipoprotein (mg/dl)[m]	110 (88 – 137)

Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; IQR, interquartile range (25%, 75%).

[a] Center for Epidemiologic Studies Depression Scale (CES-D) score 10-item version (possible range 0–30 points).

[b] Presence of depressive symptoms was defined as CES-D score ≥16.

[c] Depression was defined as presence of depressive symptoms (CES-D≥16) or use of depression medications.

[d] Binge drinking was defined as ≥5 drinks/day for ≥ 1 month; moderate-heavy drinking was defined as 3–4 drinks/day for >1 month, or ≥5 drinks/day per month; low-moderate drinking was defined as 1–2 drinks/day or 3–4 drinks/day for ≤1 month.

[e] Stimulants were defined as any use of cocaine, ecstasy, methamphetamines, or any other upper.

[f] Risky sex was defined as ≥2 partners [male or female] in the past six months.

[g] Sexually-transmitted infection was defined as any new diagnosis of herpes, syphilis, genital warts, or gonorrhea since the last visit.

[h] Persistent diabetes was defined as having a history of diabetes (fasting glucose>126 mg/dL, Hemoglobin A1c≥6.5%, or use of diabetic medications) on at least 2 occasions prior to the blood sampling date.

[i] Uncontrolled diabetes was defined as fasting glucose >126 mg/dL or Hemoglobin A1c≥ 6.5% at blood sampling.

[j] Persistent hypertension was defined as having a history of hypertension (systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or use of anti-hypertensive medications) on at least two previous occasions from the blood sampling date.

[k] Uncontrolled hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg at blood sampling.

[l] Serum cholesterol measures were obtained after fasting.

[m] 491 person-visits had available low density lipoprotein available.

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Table 2

Proportion detectable and median (IQR) of detectable biomarker concentrations, number of person-visits (*N*) = 971, Multicenter AIDS Cohort Study (MACS), 1984 – 2009

Biomarker[a]	% detectable	Median (IQR[b])
Chemokines		
CCL11	100	1,696 (1,219 – 2,419)
CXCL8	100	13.3 (8.9 – 22.9)
CXCL10	100	139 (92 – 234)
CCL2	100	508 (362 – 657)
CCL13	100	749 (580 – 991)
CCL4	99.6	145 (98 – 205)
CCL17	99.9	531 (368 – 836)
CXCL13	98.9	298 (246 – 349)
Cytokines		
Interleukin-10 (IL-10)	99.8	3.2 (2.1 – 6.3)
Interleukin-12p70 (IL-12p70)	93.5	2.6 (1.4 – 7.4)
Interleukin-6 (IL-6)	99.9	1.0 (0.7 – 1.5)
Tumor Necrosis Factor-α (TNF-α)	99.9	8.5 (6.9 – 10.6)
B-cell Activating Factor (BAFF)	100	1,970 (1,731 – 2,263)
Interferon-γ (IFN-γ)	60.5	1.3 (0.9 – 2.1)
Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF)	63.9	1.1 (0.7 – 2.1)
Interleukin-2 (IL-2)	71.8	0.7 (0.5 – 1.4)
Interleukin-1β (IL-1β)	55.7	0.5 (0.3 – 0.9)
Soluble receptors		
Soluble CD14 (sCD14), ng/mL	99.9	2,100 (1,800 – 2,500)
Soluble gp130 (sgp130), ng/mL	100	250 (230 – 290)
Soluble Interleukin 2-receptor-α (sIL2-Rα)	100	1,382 (1,138 – 1,739)
Soluble CD27 (sCD27)	100	9,067 (7,491 – 11,441)
Soluble TNF-receptor 2 (sTNF-R2)	100	2,303 (1,867 – 2,910)
C-reactive protein (CRP), mg/L	89.7	1.1 (0.5 – 2.4)

[a] Units are pg/mL unless otherwise indicated.

[b] 25th–75th percentiles.

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Table 3

Percent differences in biomarker concentrations for changes in high density and low density lipoprotein levels, Multicenter AIDS Cohort Study (MACS), 2001 – 2009

Biomarker	High density lipoprotein levels		Low density lipoprotein levels	
	Percent difference[1]	*p*-value	Percent difference[1]	p-value
CCL11	0.6	(0.809)	−1.2	(0.611)
CXCL10	−4.8	(0.114)	−3.4	(0.216)
CXCL8	−0.9	(0.717)	−2.0	(0.499)
CCL2	2.4	(0.214)	−3.1	(0.143)
CCL13	7.3	(0.009)	0.5	(0.841)
CCL4	−4.2	(0.288)	2.9	(0.303)
CXCL13	4.0	(0.004)	−3.4	(0.007)
IL-10	−6.7	(0.136)	4.4	(0.211)
IL-6	−2.6	(0.368)	2.1	(0.447)
IL-12p70	2.4	(0.771)	−3.4	(0.718)
TARC	−0.7	(0.862)	3.5	(0.324)
TNF-α	−1.7	(0.278)	−1.2	(0.470)
IL-2	10.3	(0.081)	−4.2	(0.513)
IFN-γ	−10.0	(0.074)	3.9	(0.588)
IL-1β	2.7	(0.763)	−14.4	(0.167)
GM-CSF	12.1	(0.248)	−22.2	(0.061)
BAFF	−1.3	(0.266)	−0.6	(0.588)
sCD14	−1.4	(0.448)	0.7	(0.649)
sIL-2Rα	−5.8	(0.001)	−1.7	(0.300)
sCD27	−3.2	(0.030)	−2.6	(0.087)
sgp130	2.4	(0.018)	1.0	(0.482)
sTNF-R2	−6.4	(0.000)	−3.0	(0.067)
CRP	−15.1	(0.010)	20.8	(0.002)

[1] Percent difference for a one standard deviation change in lipoprotein levels.