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Psychological factors and DNA methylation of genes related to immune/inflammatory system markers: the Normative Aging Study

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Abbreviated running title: Psychological factors and DNA methylation.

ABSTRACT

Objectives: Although psychological factors have been associated with chronic diseases such as coronary heart disease (CHD), the underlying pathways for these associations have yet to be elucidated. DNA methylation has been posited as a mechanism linking psychological factors to CHD risk. In a cohort of community-dwelling elderly men, we explored the associations between positive and negative psychological factors with DNA methylation in promoter regions of multiple genes involved in immune/inflammatory processes related to atherosclerosis.

Design: Prospective, cohort study.

Setting: Greater Boston, Massachusetts area.

Participants: Men participating in the Normative Aging Study cohort with psychological measures and DNA methylation measures, collected on one to four visits between 1999 and 2006 (mean age = 72.7 years at first visit).

Outcome measures: We examined anxiety, depression, hostility, and life satisfaction as predictors of leukocyte gene-specific DNA methylation. We estimated repeated measures linear mixed models, controlling for age, smoking, education, past history of heart disease, stroke or diabetes, % lymphocytes, % monocytes, and plasma folate.

Results: Psychological distress measured by anxiety, depression, and hostility was positively associated and happiness and life satisfaction were inversely associated with average Intercellular Adhesion Molecule-1 (*ICAM-1*) and coagulation factor III (*F3*) promoter methylation levels. There was some evidence that hostility was positively associated with toll-like receptor 2 (*TLR-2*) promoter methylation, and that life satisfaction was inversely associated with both *TLR-2* and inducible nitric oxide synthase (*iNOS*) promoter methylation. We observed less consistent and significant associations between psychological factors and average methylation for promoters of the genes for glucocorticoid receptor (*NR3C1*), interferon -γ (*IFN-γ*), and interleukin 6 (*IL-6*).

Conclusions: These findings suggest that positive and negative psychological factors affect DNA methylation of selected genes involved in chronic immune/inflammatory processes and inflammation-related endothelial dysfunction. Such epigenetic changes may represent important biological pathways that mediate the effects of psychological factors on CHD.

Keywords: psychological factors, methylation, cell adhesion molecules, *ICAM-1, F3, TLR-2*, coronary heart disease, epidemiology.

Strengths and limitations of this study

- Strengths of our study include its novel examination of multiple psychological factors (both positive and negative) in relation to DNA methylation in promoter regions of multiple genes plausibly involved in chronic immune/inflammatory processes and inflammation-related endothelial dysfunction.
- We also used repeated measures, thereby improving precision of our estimates.
- A subset of CpG sites were examined for DNA methylation within a gene promoter region, and may not necessarily have been good proxies for the all CpGs within the same region.
- The study sample was limited to an elderly, primarily white male population, and associations of psychological factors with DNA methylation may be more salient in other population sub-groups.

INTRODUCTION

Although psychological factors and clinical disorders such as anxiety and depression have been linked to a wide variety of health and disease endpoints including coronary heart disease (CHD) in epidemiological studies,[1] the mechanisms that underlie the associations with CHD have yet to be fully elucidated. CHD has been increasingly characterized as a chronic inflammatory process involving such factors as intercellular adhesion molecules [i.e., Intercellular Adhesion Molecule-1 (*ICAM-1*), Vascular Cell Adhesion Molecule-1 (*VCAM-1*)] facilitating the transendothelial migration of inflammation-related cells into vascular tissues.[2]

DNA methylation has been posited to be an intermediary mechanism by which psychological factors influence CHD risk. DNA methylation is a reversible process corresponding to the addition of methyl groups at the 5' position of cytosine rings in CpG dinucleotides to produce 5-methyl-cytosine (5mC). Decreased methylation is associated with greater RNA transcription.[3] These relatively stable epigenetic marks can modify gene expression for proteins shaping cellular signals, responses, and function. Such modifications may underlie the pathogenesis of major chronic diseases including CHD and cancer.[4-6] In humans, lower global levels of blood DNA methylation have predicted higher risks of cardiovascular diseases,[7] and alterations in the DNA methylation of specific genes have been linked to higher risks of CHD and cancer.[8,9]

Recent experimental and epidemiological evidence suggests that social/psychological exposures may contribute to the methylation of selected genes/promoters, and may thereby influence gene expression relevant to disease risk factors.[3,10-15] In rats, Weaver et al.[3] found that low levels of maternal licking and grooming led to *higher* cytosine methylation in a glucocorticoid receptor (*NR3C1*) promoter region in the brain hippocampus of offspring. Such hypermethylation is linked to lower GR expression. Because *NR3C1* up-regulation induces negative feedback in the HPA axis,[16,17] its hypothesized down-regulation with negative psychological exposures would potentially

generate pro-inflammatory stress responses. In humans, one study has reported associations between higher anxiety and depressive symptom scores in prenatal women and higher methylation of the *NR3C1* gene in newborn cord blood leukocytes and maternal blood leukocytes.[10] A study of younger to middle-aged adults found correlations between a history of childhood adversity with higher leukocyte *NR3C1* gene promoter methylation, although no correlations for anxiety and limited correlations for depression with *NR3C1* promoter methylation.[18] Distinct methylation patterns have been further observed in depressed versus not depressed individuals,[11] and lower job seniority has been linked to higher global (*Alu* line) methylation and methylation in interferon (IFN)-γ promoter regions.[12] Furthermore, individuals of low SES in early life with mothers who expressed high warmth toward them were shown to exhibit less Toll-like receptor (TLR)-stimulated production of interleukin-6 (IL-6);[19] IL-6 is an inflammatory marker that is predicted by psychosocial factors such as anxiety and depression, and is thought to be involved in the pathogenesis of cardiovascular disease.[20] Overall, these studies suggest that aspects of the social environment and mood disorders including anxiety and depression may induce epigenetic effects.[21,22] Plausibly, these epigenetic changes represent underlying common biological (e.g., immune, neuroendocrine) pathways for the putative effects of psychological factors on chronic diseases including CHD.

In a cohort of community-dwelling elderly men in the United States, we explored the associations between positive and negative psychological factors and DNA methylation in promoter regions of multiple genes involved in chronic immune/inflammatory processes and inflammation-related endothelial dysfunction. To our knowledge, this is the first study to examine a comprehensive set of psychological factors in relation to epigenetic processes plausibly related to CHD.

MATERIALS AND METHODS

Study population. The Normative Aging Study (NAS) is a longitudinal study of aging established by the US Veterans Administration. The original cohort was recruited between 1961 and 1970, and consisted of 2,280 community-dwelling men from the greater Boston, Massachusetts area aged 21–80 years who were free of known chronic medical conditions at enrollment.[23] Every three to five years, study participants have undergone routine physical examinations and laboratory tests, and responded to surveys on medical history, lifestyle factors, and psychological factors.

The present study analyzed data on men participating in the NAS cohort with psychological measures and DNA methylation measures (average of 2.2 measures/individual), collected on between one to four visits between 1999 and 2006. During this time period, 765 study participants provided at least one whole blood sample that was used to measure DNA methylation. Because for some subjects the extracted DNA was not sufficient in quantity to conduct methylation assays for all genes and due to some assay failures, the total numbers of men in whom there were assays corresponding to promoter regions of different genes varied.[24]

Outcome variables. The average and position-specific levels of methylation in promoter regions of seven genes [toll-like receptor 2 (*TLR-2*), coagulation factor III (*F3*), glucocorticoid receptor (*NR3C1*), intercellular adhesion molecule-1 (*ICAM-1*), interferon-γ (*IFN-γ*), interleukin 6 *(IL-6*), inducible nitric oxide synthase (*iNOS*)] were analyzed as outcomes in separate models.

These genes were selected based on past evidence for associations of: 1) proteins coded by these genes in animal and/or human studies of atherosclerosis or the pathophysiology of heart disease; 2) psychological factors with methylation of promoters of the genes; and 3) psychological factors with peripheral blood levels of the markers expressed by these genes. For instance, for the first selection criterion, both serum *ICAM-1* and *IL-6* levels have independently predicted CHD risk in prospective studies after controlling for demographic/socioeconomic and traditional CHD risk factors.[25,26] In the

Introduction, we cited studies suggesting linkages between psychological exposures and the methylation of *NR3C1* and *IFN-γ* promoters, which in turn might explain chronic inflammatory processes characterizing diseases such as CHD. As an example for the third selection criterion, lower early-life socioeconomic status (SES) has been linked to greater expression of both *NR3C1* and *TLR* receptor mRNA in leukocytes.[27]

DNA was extracted from stored frozen buffy coat of 7 mL whole blood, using the QiAmp DNA blood kits (QIAGEN, Hilden, Germany). 500 ng DNA (concentration 50 ng/µl) was treated using EZ DNA Methylation-GoldTM Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Final elution was performed with 30 µl of M-Elution Buffer.

CpG dinucleotide-rich promoter regions were identified using the Genomatix Software Suite (Genomatix, Germany). Promoters without any assigned transcripts were excluded. To the best of our knowledge, there were no DNA methylation assays for the genes analyzed that were already published. Therefore, we developed new pyrosequencing assays by selecting amplicons in promoter CpG-rich areas. For each gene, the PCR-pyrosequencing primer (more than 20 base pairs long) of the highest available quality that was associated with one of the promoters was designed using specialized software (PSQ Assay Design, Biotage, Sweden). The fractions of CpG sites examined by gene were as follows: *TLR-2* (5/49); *F3* (5/78); *NR3C1* (1/7); *ICAM-1* (5/69); *IFN-γ* (2/8); *IL-6* (2/18); *iNOS* (2/8). We did not assay higher proportions of CpG sites due to inherent limitations of the method applied i.e., we excluded PCR amplicons with 350 or fewer base pairs, primers that avoided CpGs, and target sequences of 40 or fewer base pairs. We did not have additional information about the CpGs that were analyzed (e.g., for NR1C3), including their functionality or their proximity to transcription factor-binding sites or other important sequences. Supplementary Table 1 lists the specific CpG positions for DNA methylation that we measured within specified promoter regions for each gene.

The degree of methylation was calculated as the percentage of methylated cytosine residues divided by the sum of methylated and unmethylated cytosine residues (%5mC) in each sample. Built-in controls were used to verify bisulfite conversion efficiency. Each sample was tested twice for each marker to improve statistical power and precision. The average of the replicates was used.

Predictor variables. We used data on anxiety and depression measured through the Brief Symptom Inventory (BSI), a self-administered 53-item questionnaire of nine primary psychological symptom dimensions (anxiety, depression, hostility, interpersonal sensitivity, obsessive-compulsive, paranoid ideation, phobic anxiety, psychoticism, somatization) experienced by the respondent over the previous 30 days; the BSI was included as part of the Health and Social Behavior Survey in the NAS starting in 1985.[23,28] Happiness (based on the single item "How happy are you right now?") and life satisfaction (based on the 11-item version of the Life Satisfaction Inventory-A[29]) were also examined as predictor variables. Higher life satisfaction scale scores corresponded to higher self-reported life satisfaction; higher scores on the other scales reflected higher negative psychological symptoms. All psychological measures were analyzed as continuous. Internal consistency reliability (Cronbach's α) values for the anxiety, depression, hostility, and life satisfaction scales were all acceptably high (>0.70).

Covariates. Model covariates consisted of the age at first visit in or after 1999 (years), smoking (pack-years of smoking), education (>high school, ≤high school), history of CHD or stroke prior to 1999, history of diabetes prior to 1999, % lymphocytes, % monocytes, and plasma folate levels. Previous evidence suggests that leukocyte composition is related to DNA methylation,[30] and that folate is a source of methyl groups and folate depletion leads to lower blood DNA methylation.[31] Because 98% of the sample was White, we did not adjust for race/ethnicity.

Statistical analysis. We first calculated descriptive statistics (mean, range, percentages for psychological factors and covariates, mean percentage methylation for gene-specific promoter

methylation) based on study participants with measures of ICAM-1 promoter region methylation, which showed several significant associations.

We then constructed a Pearson correlation coefficient matrix for the psychological factors and a correlation coefficient matrix for the methylation outcomes.

To examine the associations between the psychological factors and the methylation outcomes, we next estimated repeated measures linear mixed models (equivalent to random intercept models) to account for up to four repeated measures, using a first-order autoregressive covariance structure (in which a decreasing correlation of standard errors over time was modeled). The log-likelihood fit statistics for the models indicated better model fits than those for the corresponding models using a compound symmetry covariance structure; unstructured covariance structure models did not converge. Because we assumed a short latency period for methylation changes, we modeled each psychological factor as a predictor of gene-specific methylation measured on the same visit (averaged across cytosines in CpG sites within the promoter region, varying from one CpG site for the *NR3C1* gene to five CpG sites for the *F3* gene according to the density of CpG sites in the sequence amplified within the promoter region). In addition, we noted the associations between selected covariates (age, smoking, income/education) and methylation.

For primary associations significant at the 5% level, we further tested for dose-response relationships, by grouping the respective psychological factor into meaningful and/or equally-sized categories where possible. A dose-response relationship would lend support to a casual association.[32] A linear test for trend was performed by converting the categories into an ordinal variable and noting its corresponding p value.

We further examined the associations between psychological factors and serum ICAM-1, to examine whether similar relationships were present as between the psychological factors and

ICAM-1 promoter methylation levels (because the latter would be expected to be inversely related to ICAM-1 expression).

Finally, because of the known association between aging and methylation, we repeated the analyses using age^2 as an additional covariate to saturate the model for an age effect and found comparable results (data not shown).

All tests were two-tailed with a 5% significance level. All analyses were conducted using SAS Version 9.1 (SAS Institute, Cary, NC).

All participants gave written informed consent. This research was approved by the human subjects committees of the Boston VA Medical Center and the Harvard School of Public Health.

RESULTS

Characteristics of study sample. Table 1 shows descriptive characteristics of the study sample based on 616 men with measures of ICAM-1 promoter region methylation. We present characteristics for this sample because several of the corresponding associations with ICAM-1 methylation were significant among the different gene promoter regions analyzed. The sample had a mean age of 72.5 years (range 56-100 years) at first visit. Approximately one-third (34.1%) attained no more than high school education and over two-thirds had previously smoked, with an average of 21.8 pack-years of smoking (Table 1). These characteristics were similar to those of the larger cohort of men with visits between 1999 and 2006 including men with missing observations for methylation (n = 1,121 men: mean age 71.7 years, % with less than high school education = 35.9; mean pack-years of smoking = 21.6). After listwise deletion of missing data in respective models, the sizes of analytic samples ranged from 481 to 669 men. Missing gene-specific methylation data ranged from 5.4% (*IFN-γ*) to 23.8% (*iNOS*), due to the presence of assay failures and the lack of sufficient DNA, which disproportionately affected genes that were tested later in the order (i.e.,

iNOS, *ICAM-1*). Missing model covariate data ranged collectively from 3.1% to 3.5%. Missing psychological factor data ranged from 3.7% (happiness) to 10.8% (life satisfaction) in the respective model (Supplementary Table 2). Mean leukocyte methylation levels within promoter regions ranged from 2.2% 5mC (*OGG* gene) to 84.8% 5mC (IFN-γ gene); none of the distributions was highly skewed (Table 1).

Anxiety, depression, and hostility scale scores were significantly positively correlated with one another, and were nearly all significantly inversely correlated with happiness and life satisfaction scores (all $|r|>0.3$ and $p<0.01$; Table 2). By contrast, none of the methylation outcomes were moderately to strongly correlated with one another (all $|r|<0.3$; data not shown).

Associations between psychological factors and average DNA methylation. Table 3 shows the multivariate-adjusted coefficient estimates from repeated measures models. Negative psychological factors were related to higher average methylation in *ICAM-1* promoter regions (with the associations for anxiety significant at the 0.10 level and for depression significant at the 0.05 level). Happiness was significantly inversely associated with *ICAM-1* promoter methylation. Depression was significantly positively associated and happiness and life satisfaction were significantly inversely associated with average methylation in *F3* promoter regions, respectively. For *TLR-2* promoter methylation, all negative psychological factors showed positive relations (with the association for hostility significant at the 0.10 level) and both positive psychological factors showed inverse relations (with the association for life satisfaction significant at the 0.05 level). For *iNOS* promoter methylation, all negative psychological factors showed inverse relations and both positive psychological factors showed positive relations. However, only the association for life satisfaction was significant at the 0.10 level. For *NR3C1* promoter methylation, depression, hostility, happiness, and life satisfaction all exhibited positive and non-significant associations.

Likewise, psychological factors were inconsistently and non-significantly related to higher methylation in the promoter regions for *IFN-γ* and *IL-6*.

For all associations significant at the 0.05 level, we further identified monotonic dose-response relationships, with categories of higher scores of the psychological factors being associated with stronger associations. Tables 4 and 5 show the coefficient estimates across categories as well as the p values from the tests for linear trend across categories; these p values were significant at the 0.05 level for *F3* promoter methylation and at the 0.10 level for *ICAM-1* promoter region methylation, respectively.

In all models, pack-years of smoking significantly predicted higher average methylation levels in the gene-specific promoter regions. Age was non-significantly inversely associated with methylation. Additional adjustment for household income (with lower income being non-significantly positively associated with methylation) did not alter the main results (data not shown).

Associations between psychological factors and serum ICAM-1. No psychological factors were associated with serum ICAM-1 levels (for anxiety: β=5.11, p=0.51; other psychological factors exhibited similar associations). ICAM-1 methylation levels and serum ICAM-1 levels were uncorrelated (r = -0.04).

DISCUSSION

In this study of community-dwelling elderly adult men, we found consistent associations between both positive and negative psychological factors with higher average leukocyte DNA methylation in *ICAM-1* promoter regions and in *F3* promoter regions. There was some evidence that hostility was positively associated with *TLR-2* promoter methylation, and that life satisfaction was inversely associated with both *TLR-2* and *iNOS* promoter methylation. We observed less consistent and

significant associations between psychological factors and average methylation for promoters of the genes for *NR3C1*, *IFN-γ*, and *IL-6*.

Our main findings were generally robust across multiple Brief Symptom Inventory (BSI) component scales. While this may stem from similarities across component scale measures, results using very different scales (e.g., life satisfaction) were qualitatively consistent. Moreover, smoking has been linked to pro-inflammatory states and atherosclerosis,[33] and the direction of the associations for smoking with hypermethylation of *ICAM-1* promoter regions matched those for negative psychological factors, providing support that the associations were not simply attributable to chance. Our findings were furthermore robust to the adjustment of the presence of CHD, stroke, and diabetes, countering underlying co-morbidities/health selection as alternative explanations for the main findings.

Higher circulating levels of serum *ICAM-1* have been previously independently linked to modest risks of CHD after adjusting for key covariates such as SES.[34-36] Notably, we found no association between psychological factors and serum *ICAM-1*. Along with the presence of associations between psychological factors and *ICAM-1* promoter methylation, this could be explained by the fact that serum *ICAM-1* is derived from multiple sources (vascular endothelium, macrophages, lymphocytes), consistent with the absence of a correlation between leukocyte *ICAM-1* methylation and serum *ICAM-1*. Past investigations of the Normative Aging Study have likewise found no association between serum *ICAM-1* and global (*LINE-1*) leukocyte methylation levels.[37] Whether methylation of *ICAM-1* in white blood cells predicts serum *ICAM-1* levels derived solely from white blood cells (vs. other sources), and whether this *ICAM-1* independently contributes to higher risks of CHD should be explored in future studies.

Atherosclerosis is a chronic inflammatory process involving the infiltration of leukocytes and smooth muscle cells into the extravascular space, mediated in part by adhesion molecules. *ICAM-1*

plays a pivotal role in the adhesion of leukocytes to the endothelium.[38-40] Given evidence that psychological factors are risk factors for atherosclerosis,[1] we propose two explanations for negative psychological factors being linked to higher *ICAM-1* promoter region methylation in leukocytes. The first posited mechanism is *competitive binding*. In rats, recombinant induction of higher serum *ICAM-1* levels reduces leukocyte adhesion, plausibly by sterically inhibiting alternative ICAM-1 binding.[41] *ICAM-1* is also known to compete with *ICAM-2* in their contributions to pro-inflammatory environments. Low leukocyte membrane levels of *ICAM-1* resulting from higher methylation of the ICAM-1 promoter may contribute to decreased binding of leukocyte *ICAM-1* to integrin receptors on the cell membranes of these leukocytes. Through competitive binding, lower levels of leukocyte *ICAM-1* could thus facilitate vascular endothelial cell *ICAM-1* binding to leukocytes. Higher methylation of leukocyte *ICAM-1* may then be associated with greater binding of leukocytes to endothelial cells and their transmigration into extravascular tissues. The second posited mechanism is *cellular signaling*, with *ICAM-1* being known to function via signal transduction[42,43] Low binding of leukocyte *ICAM-1* to its cell membrane integrins could trigger a cascade of pro-inflammatory mediators and signal endothelial cells to release *ICAM-1*,[40,44-46] and could thereby stimulate *ICAM-1* leukocyte binding to vascular endothelial cells. Hence, through signaling mechanisms, low leukocyte *ICAM-1* levels could induce leukocyte migration into vascular endothelial tissues. Future biological studies (e.g., animal experiments which manipulate distress or other exposures) should further investigate and test these two hypothesized pathways.

Depression was positively associated and happiness and life satisfaction were each inversely associated with higher *F3* promoter methylation in leukocytes (which in turn would be linked to reduced leukocyte F3 expression). Some evidence suggests that the major source of *F3* in arterial thrombosis is the vascular wall rather than monocytes,[47] although monocyte *F3* also contributes to inflammation and thrombosis. *F3*, also known as Tissue Factor, has been shown to be involved in

cellular signaling and inflammatory pathways.[48,49] Like the hypothesis for *ICAM-1*, low leukocyte *F3* levels via signaling pathways may promote inflammatory states through greater vascular *F3* levels.

Furthermore, hostility was positively associated and life satisfaction was inversely associated with higher *TLR-2* promoter methylation, which would imply lower *TLR-2* expression. These findings appear contrary to the hypothesized role that *TLR-2* plays in atherosclerosis.[50,51] Nonetheless, there is some evidence to suggest that *TLR-2* promoter hypermethylation is present in chronic inflammatory processes such as periodontitis.[52] In addition, it has been suggested that the inflammatory process itself may induce cytosine damage and aberrant methylation patterns, including hypermethylation.[53] Furthermore, the association of negative psychological states such as hostility with decreased expression of TLR-2 may signify suppression of the immune system; this is consistent with observed relationships between stress and immune suppression in other studies.[54]

We found no associations between psychological factors and leukocyte *NR3C1* promoter methylation. Previous studies in humans have yielded conflicting results. For example, an investigation in prenatal women using clinically-administered (Hamilton Rating) scales of anxiety and depression and a self-administered (Edinburgh Postnatal Depression) scale of depression observed associations between higher maternal anxiety and depressive symptom scores and methylation of CpGs within the promoter and exon 1F of the *NR3C1* gene (homologous to the l_7 region of the rat *NR3C1* gene) in maternal blood leukocytes.[10] A study of men and women aged 18-59 reported correlations between a history of childhood adversity with higher leukocyte *NR3C1* gene promoter methylation, yet found no correlations for anxiety (using the State-Trait Anxiety Inventory) and only limited correlations for depression (using the Inventory for Depressive Symptoms) with GR promoter methylation (at 0 of 13 CpG sites and 2 of 13 CpG sites, respectively).[18] Meanwhile, a recent brain post-mortem study in adults found no hippocampal GR

promoter methylation differences between those clinically diagnosed with major depression versus controls.[55]

Strengths of our study include its examination of multiple psychological factors (both positive and negative) and its novel exploration of DNA methylation in promoter regions of multiple genes plausibly involved in chronic immune/inflammatory processes and inflammation-related endothelial dysfunction; its reliance on a community-based sample which strengthens generalizability of our findings; and its use of repeated measures, thereby improving precision of our estimates. We further tested for and confirmed linear dose-response relationships, which support the presence of causal associations.

There were several limitations to our study. First, we examined DNA methylation at a subset of CpG sites within a gene promoter region. The inability to assay high proportions given methodological limitations could have led us to the omission of some relevant CpG sites. The analyzed CpGs (selected based on aforementioned methodological limitations) may not necessarily have been good proxies for the rest of the CpGs within the same regions. Second, differences in results from previous studies, particularly for *NR3C1* methylation, might also stem from the measurement of methylation in peripheral blood rather than hippocampal tissue; methylation effects may be tissue specific.[18,56] Third, due to the multiple associations examined, the multiple comparisons problem, whereby multiple comparisons may increase the presence of significant associations by chance, cannot be ruled out. Fourth, while the null associations for methylation in promoter regions of several genes including *NR3C1*, *IFN-γ*, and *IL-6* could reflect the true absence of associations, they could also possibly be attributed to selection bias due to attrition or missing methylation data, as suggested by demographic (age, education) differences in those analyzed versus the NAS cohort in 1985 when the BSI was first administered. For instance, those with a stronger association between the psychological factors and methylation may have either died or

have been lost to follow-up, leading to attenuated and null associations in the analyzed data. With respect to the varying sample sizes between analytic samples for genes examined, the mechanism of missing data due to insufficient DNA and assay failures was plausibly missing completely at random (MCAR), and entirely unrelated to the levels of methylation of a particular sequence of DNA.[24] Under the MCAR mechanism, the listwise deletion method that we applied should be valid.[57] In support of the MCAR assumption being met, we determined that those participants with and without missing methylation data for each gene were generally comparable on demographic characteristics (mean age, distribution of education), mean pack-years of smoking, and mean anxiety and depression scores. Finally, the presence of null associations may in part be due to the study sample being limited to an elderly, primarily white male population. Effects of psychological factors on DNA methylation may be more salient in other population sub-groups, or at earlier, sensitive time-points over the life-course. Future studies should extend examination of these associations to younger adults, older women, and members of other racial/ethnic groups.

In summary, our study primarily suggests novel relations between positive and negative psychological factors and methylation of ICAM-1 promoter regions and linkages with F3 gene methylation, and to a lesser extent associations with *TLR-2* promoter methylation. Confirming these findings in other populations and settings may yield a better understanding of the epigenetic mechanisms by which psychological factors influence CHD and other major chronic disease outcomes.

Contributorship Statement

DK, LDK, and JS conceived and designed the study. AB, DS, AS, LT, LC, PV, and JS gathered data. DK performed all data analyses, and drafted the manuscript. DK, LDK, AB, and JS revised the manuscript for important intellectual content.

Competing Interests

There are no competing interests.

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Data Sharing Statement

Data are from the Normative Aging Study, whose restricted data are available for researchers who meet the criteria.

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Table 1. Descriptive statistics (mean values with ranges in parentheses; percentages) for samples analyzed with respective characteristic and ICAM-1 promoter methylation (n ranging from 538 to 577 men)*

Mean age in yrs at first visit in 1999	72.5 (56-100)
% ≤High school	34.1
% White	98.0
% with CHD/stroke/diabetes	33.3
Smoking in pack-years	21.8 (0-131)
Anxiety	0.20 (0-2.83)
Depression	0.20 (0-3.33)
Hostility	0.21 (0-3.00)
Happiness	7.39 (1-9)
Life satisfaction	7.88 (0-11)
DNA methylation in gene promoter regions (%)	
TLR-2	3.1 (0-8.9)
F3	2.3 (0-14.8)
NR3C1	47.0 (14.7-72.8)
ICAM-1	4.4 (1.7-16.1)
IFN-γ	84.4 (30.9-95.7)
IL-6	43.7 (10.3-86.6)
iNOS	69.7 (24.5-87.2)

Table 2. Pearson correlation coefficients between psychological factors.*

	Anxiety	Depression	Hostility	Happiness	Life satisfaction
Anxiety	1.00	0.76 (n=611)	0.67 (n=611)	-0.32 (n=612)	-0.31 (n=578)
Depression		1.00	0.63 (n=609)	-0.46 (n=611)	-0.42 (n=577)
Hostility			1.00	-0.30 (n=610)	-0.28 (n=577)
Happiness				1.00	0.58 (n=598)
Life satisfaction					1.00

*For men with observations for methylation in ICAM-1 promoter regions.
P<0.01 for all correlations.

Table 3. Coefficient estimates (95% CI) for multivariate associations between psychological factors and average methylation in gene promoter regions, from repeated measures models.

	Gene						
	TLR-2	*F3*	*NR3C1*	*ICAM-1*	*IFN-γ*	*IL-6*	*iNOS*
Anxiety	0.07 (-0.17, 0.32) n=558; 833 obs	0.17 (-0.05, 0.40) n=607; 909 obs	-0.42 (-1.54, 0.71) n=581; 924 obs	0.34** (-0.03, 0.72) n=548; 831 obs	0.50 (-0.41, 1.40) n=640; 1069 obs	0.36 (-1.75, 2.47) n=636; 1077 obs	-0.82 (-2.28, 0.64) n=499; 729 obs
Depression	0.08 (-0.15, 0.30) n=554; 825 obs	0.34* (0.14, 0.55) n=605; 904 obs	0.22 (-0.76, 1.21) n=579; 919 obs	0.38* (0.04, 0.72) n=546; 826 obs	0.21 (-0.62, 1.04) n=638; 1064 obs	-0.12 (-2.07, 1.83) n=634; 1071 obs	-0.60 (-1.93, 0.73) n=496; 723 obs
Hostility	0.22** (-0.04, 0.49) n=554, 828 obs	0.18 (-0.06, 0.42) n=603; 905 obs	0.20 (-1.00, 1.40) n=578; 921 obs	0.20 (-0.19, 0.60) n=545; 828 obs	0.39 (-0.56, 1.34) n=636; 1066 obs	-0.54 (-2.74, 1.66) n=632; 1074 obs	-0.34 (-1.82, 1.14) n=497; 727 obs
Happiness	-0.02 (-0.09, 0.05) n=582; 867 obs	-0.10* (-0.16, -0.04) n=636; 952 obs	0.12 (-0.17, 0.41) n=608; 967 obs	-0.10* (-0.22, -0.003) n=577; 871 obs	0.04 (-0.20, 0.28) n=669; 1117 obs	-0.38 (-0.95, 0.19) n=666; 1128 obs	0.07 (-0.33, 0.47) n=523; 760 obs
Life Satisfaction	-0.05* (-0.09, -0.01) n=539; 808 obs	-0.06* (-0.10, -0.03) n=590; 885 obs	0.09 (-0.09, 0.26) n=563; 895 obs	-0.02 (-0.08, 0.04) n=538; 813 obs	-0.04 (-0.19, 0.10) n=619; 1036 obs	0.15 (-0.18, 0.49) n=615; 1045 obs	0.20** (-0.02, 0.43) n=481; 698 obs

Associations between each psychological factor and average levels of methylation across CpG sites within gene promoter regions examined in separate models. All models adjusted for age, smoking status, educational attainment, history of CHD or stroke prior to 1999, history of diabetes prior to 1999, % lymphocytes, % monocytes, and plasma folate.
*P<0.05.** P<0.10.

Table 4. Coefficient estimates from repeated measures models for multivariate associations between categorized scale values of depression, happiness, life satisfaction and *F3* promoter methylation (n = 658 men, 988 observations).

		Coefficient Estimate	95% CI	P value
Depression				
	0	-	-	-
	0.01-0.4	-0.13	-0.34, 0.09	0.24
	>0.4	0.33	0.10, 0.56	0.005
				$P_{trend} = 0.03$
Happiness				
	1-4 (unhappy)	-	-	
	5-7	-0.20	-0.54, 0.14	0.24
	8-9 (happy)	-0.51	-0.85, -0.18	0.003
				$P_{trend} <.001$
Life satisfaction				
	0-5	-	-	-
	6-8	-0.28	-0.49, -0.06	0.01
	9-11	-0.40	-0.60, -0.20	<0.001
				$P_{trend} <.001$

F3 methylation values corresponded to the average levels of methylation across CpG sites within the *F3* promoter region.
All models adjusted for age, smoking status, educational attainment, history of CHD or stroke prior to 1999, history of diabetes prior to 1999, % lymphocytes, % monocytes, and plasma folate.

Table 5. Coefficient estimates from repeated measures models for multivariate associations between categorized scale values of depression and happiness and *ICAM-1* promoter methylation (n = 600 men, 906 observations)

		Coefficient Estimate	95% CI	P value
Depression				
	0	-	-	-
	0.01-0.4	0.19	-0.16, 0.55	0.29
	>0.4	0.30	-0.09, 0.70	0.13
				$P_{trend} = 0.09$
Happiness				
	1-4 (not happy)	-	-	-
	5-7	-0.21	-0.76, 0.34	0.46
	8-9 (happy)	-0.42	-0.97, 0.13	0.13
				$P_{trend} = 0.06$

ICAM-1 methylation values corresponded to the average levels of methylation across CpG sites within the *ICAM-1* promoter region.
All models adjusted for age, smoking status, educational attainment, history of CHD or stroke prior to 1999, history of diabetes prior to 1999, % lymphocytes, % monocytes, and plasma folate.

Supplementary Table 1. Location of the CpG position and the promoter region for each gene.*

Gene	Chromosome	Promoter		CpG Positions for measured DNA methylation				
		Start	End	Position 1	Position 2	Position 3	Position 4	Position 5
TLR-2	4	154824391	154824991	154824709	154824713	154824715	154824723	154824727
F3	1	94779671	94780502	94779947	94779950	94779956	94779958	94779974
NR3C1	5	142760496	142761097	142760565				
ICAM-1	19	10242017	10242937	10242236	10242225	10242218		
IFN-γ	12	66839561	66840293	66840192	66840186			
Il-6	7	22732791	22733685	22733847	22733841			
iNOS	17	23149861	23150461	23149929	23149936			

*NCBI build 36.1 was used as the reference of the human genome in this study.

Supplementary Table 2. Numbers and percentages of missing men for methylation in the promoter region for each gene, for model covariates, and respective psychological factors (n = 765 men without excluding those with missing values).

Psychological Factor	*TLR-2*	*F3*	*NR3C1*	*ICAM-1*	*IFN-γ*	*IL-6*	*iNOS*
Anxiety	Missing methylation: n=123; 16.1% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=74; 9.7% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=100; 13.1% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=133; 17.4% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=41; 5.4% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=45; 5.9% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=182; 23.8% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)
Depression	Missing methylation: n=125; 16.3% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=74; 9.7% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=100; 13.1% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=133; 17.4% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=41; 5.4% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=45; 5.9% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=183; 23.9% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)
Hostility	Missing methylation: n=123; 16.1% Missing	Missing methylation: n=74; 9.7% Missing covariates:	Missing methylation: n=99; 12.9% Missing	Missing methylation: n=132; 17.3% Missing	Missing methylation: n=41; 5.4% Missing	Missing methylation: n=45; 5.9% Missing	Missing methylation: n=180; 23.5% Missing

	covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	n=27 (3.5%) Missing hostility: n=61 (8.0%)	covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)
Happiness	Missing methylation: n=128; 16.7% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=74; 9.7% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=102; 13.3% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=133; 17.4% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=41; 5.4% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=44; 5.8% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=187; 24.4% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)
Life satisfaction	Missing methylation: n=119; 15.6% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=68; 8.9% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=95; 12.4% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=120; 15.7% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=39; 5.1% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=43; 5.6% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=177; 23.1% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Psychological factors and DNA methylation of genes related to immune/inflammatory system markers: the VA Normative Aging Study

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Psychological factors and DNA methylation of genes related to immune/inflammatory system markers: the VA Normative Aging Study

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Abbreviated running title: Psychological factors and DNA methylation.

ABSTRACT

Objectives: Although psychological factors have been associated with chronic diseases such as coronary heart disease (CHD), the underlying pathways for these associations have yet to be elucidated. DNA methylation has been posited as a mechanism linking psychological factors to CHD risk. In a cohort of community-dwelling elderly men, we explored the associations between positive and negative psychological factors with DNA methylation in promoter regions of multiple genes involved in immune/inflammatory processes related to atherosclerosis.

Design: Prospective cohort study.

Setting: Greater Boston, Massachusetts area.

Participants: Samples of 538 to 669 men participating in the Normative Aging Study cohort with psychological measures and DNA methylation measures, collected on 1-4 visits between 1999 and 2006 (mean age = 72.7 years at first visit).

Outcome measures: We examined anxiety, depression, hostility, and life satisfaction as predictors of leukocyte gene-specific DNA methylation. We estimated repeated measures linear mixed models, controlling for age, smoking, education, history of heart disease, stroke or diabetes, % lymphocytes, % monocytes, and plasma folate.

Results: Psychological distress measured by anxiety, depression, and hostility was positively associated and happiness and life satisfaction were inversely associated with average Intercellular Adhesion Molecule-1 (*ICAM-1*) and coagulation factor III (*F3*) promoter methylation levels. There was some evidence that hostility was positively associated with toll-like receptor 2 (*TLR-2*) promoter methylation, and that life satisfaction was inversely associated with *TLR-2* and inducible nitric oxide synthase (*iNOS*) promoter methylation. We observed less consistent and significant associations between psychological factors and average methylation for promoters of the genes for glucocorticoid receptor (*NR3C1*), interferon -γ (*IFN-γ*), and interleukin 6 (*IL-6*).

Conclusions: These findings suggest that positive and negative psychological factors affect DNA methylation of selected genes involved in chronic immune/inflammatory processes and inflammation-related endothelial dysfunction. Such epigenetic changes may represent biological pathways that mediate the effects of psychological factors on CHD.

Keywords: psychological factors, methylation, cell adhesion molecules, *ICAM-1, F3, TLR-2*, coronary heart disease, epidemiology.

Strengths and limitations of this study

- Strengths of our study include its novel examination of multiple psychological factors (both positive and negative) in relation to DNA methylation in promoter regions of multiple genes plausibly involved in chronic immune/inflammatory processes and inflammation-related endothelial dysfunction.
- We also used repeated measures, thereby improving precision of our estimates.
- A subset of CpG sites were examined for DNA methylation within a gene promoter region, and may not necessarily have been good proxies for the all CpGs within the same region.
- The study sample was limited to an elderly, primarily white male population, and associations of psychological factors with DNA methylation may be more salient in other population sub-groups.

INTRODUCTION

Although psychological factors and clinical disorders such as anxiety and depression have been linked to a wide variety of health and disease endpoints including coronary heart disease (CHD) in epidemiological studies,[1-3] the mechanisms that underlie the associations with CHD have yet to be fully elucidated. CHD has been increasingly characterized as a chronic inflammatory process involving such factors as intercellular adhesion molecules [i.e., Intercellular Adhesion Molecule-1 (*ICAM-1*), Vascular Cell Adhesion Molecule-1 (*VCAM-1*)] facilitating the transendothelial migration of inflammation-related cells into vascular tissues.[4]

DNA methylation may be an intermediary mechanism by which psychological factors influence CHD risk. DNA methylation is a reversible process corresponding to the addition of methyl groups at the 5' position of cytosine rings in CpG dinucleotides to produce 5-methyl-cytosine (5mC). DNA methylation is involved in regulation of gene expression and in several genes, lower methylation has been associated with increased mRNA expression.[5] These relatively stable epigenetic marks can modify gene expression for proteins shaping cellular signals, responses, and function. Such modifications may underlie the pathogenesis of major chronic diseases including CHD and cancer.[6-8] In humans, lower levels of blood LINE-1 DNA methylation have predicted higher risks of cardiovascular diseases,[9] and alterations in the DNA methylation of specific genes have been linked to higher risks of CHD and cancer.[10,11]

Recent experimental and epidemiological evidence suggests that social/psychological exposures may contribute to the methylation of selected genes/promoters, and may thereby influence gene expression relevant to disease risk factors.[5,12-17] In rats, Weaver et al.[5] found that low levels of maternal licking and grooming led to *higher* cytosine methylation in a glucocorticoid receptor (*NR3C1*) promoter region in the brain hippocampus of offspring. Such hypermethylation is linked to lower GR expression. Because *NR3C1* up-regulation induces negative feedback in the

hypothalamic-pituitary-adrenal (HPA) axis,[18,19] its hypothesized down-regulation with negative psychological exposures would potentially generate pro-inflammatory stress responses. In humans, one study has reported associations between higher anxiety and depressive symptom scores in prenatal women and higher methylation of the *NR3C1* gene in newborn cord blood leukocytes and maternal blood leukocytes.[12] A study of younger to middle-aged adults found correlations between a history of childhood adversity with higher leukocyte *NR3C1* gene promoter methylation, although no correlations for anxiety and limited correlations for depression with *NR3C1* promoter methylation.[20] Distinct methylation patterns have been further observed in depressed versus not depressed individuals,[13] and lower job seniority has been linked to higher global (*Alu* line) methylation and methylation in interferon (IFN)-γ promoter regions.[14] Furthermore, individuals of low socioeconomic status (SES) in early life with mothers who expressed high warmth toward them were shown to exhibit less Toll-like receptor (TLR)-stimulated production of interleukin-6 (IL-6);[21] IL-6 is an inflammatory marker that is predicted by psychosocial factors such as anxiety and depression, and is thought to be involved in the pathogenesis of cardiovascular disease.[22] Overall, these studies suggest that aspects of the social environment and mood disorders including anxiety and depression may induce epigenetic effects.[23,24] Plausibly, these epigenetic changes represent underlying common biological (e.g., immune, neuroendocrine) pathways for the putative effects of psychological factors on chronic diseases including CHD.

In a cohort of community-dwelling elderly men in the United States, we explored the associations between positive and negative psychological factors and DNA methylation in promoter regions of multiple genes involved in chronic immune/inflammatory processes and inflammation-related endothelial dysfunction. These genes include the ones for the proteins noted above and for *F3* (also known as Tissue Factor) and *iNOS,* that have been shown to be involved in chronic inflammatory pathways and have been previously linked to chronic inflammatory conditions.[25-30]

To our knowledge, this is the first study to examine a comprehensive set of psychological factors in relation to epigenetic processes plausibly related to CHD.

MATERIALS AND METHODS

Study population. The Normative Aging Study (NAS) is a longitudinal study of aging established by the US Veterans Administration. The original cohort was recruited between 1961 and 1970, and consisted of 2,280 community-dwelling men from the greater Boston, Massachusetts area aged 21–80 years who were free of known chronic medical conditions at enrollment.[31] Every three to five years, study participants have undergone routine physical examinations and laboratory tests, and responded to surveys on medical history, lifestyle factors, and psychological factors.

The present study analyzed data on men participating in the NAS cohort with psychological measures and DNA methylation measures (average of 2.2 measures/individual), collected on between one to four visits between 1999 and 2006. During this time period, 765 study participants provided at least one whole blood sample that was used to measure DNA methylation. Because for some subjects the extracted DNA was not sufficient in quantity to conduct methylation assays for all genes and due to some assay failures, the total numbers of men in whom there were assays corresponding to promoter regions of different genes varied.[32]

Outcome variables. The average and position-specific levels of methylation in promoter regions of seven genes [toll-like receptor 2 (*TLR-2*), coagulation factor III (*F3*), glucocorticoid receptor (*NR3C1*), intercellular adhesion molecule-1 (*ICAM-1*), interferon-γ (*IFN-γ*), interleukin 6 *(IL-6*), inducible nitric oxide synthase (*iNOS*)] were analyzed as outcomes in separate models.

These genes were selected based on past evidence for associations of: 1) proteins coded by these genes in animal and/or human studies of atherosclerosis or the pathophysiology of heart disease; 2) psychological factors with methylation of promoters of the genes; and 3) psychological factors with

peripheral blood levels of the markers expressed by these genes. For instance, for the first selection criterion, both serum *ICAM-1* and *IL-6* levels have independently predicted CHD risk in prospective studies after controlling for demographic/socioeconomic and traditional CHD risk factors.[33,34] In the Introduction, we cited studies suggesting linkages between psychological exposures and the methylation of *NR3C1* and *IFN-γ* promoters, which in turn might explain chronic inflammatory processes characterizing diseases such as CHD. As an example for the third selection criterion, lower early-life socioeconomic status (SES) has been linked to greater expression of both *NR3C1* and *TLR* receptor mRNA in leukocytes.[35]

DNA was extracted from stored frozen buffy coat of 7 mL whole blood, using the QiAmp DNA blood kits (QIAGEN, Hilden, Germany). 500 ng DNA (concentration 50 ng/µl) was treated using EZ DNA Methylation-GoldTM Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Final elution was performed with 30 µl of M-Elution Buffer.

CpG dinucleotide-rich promoter regions were identified using the Genomatix Software Suite (Genomatix, Germany). Promoters without any assigned transcripts were excluded. To the best of our knowledge, there were no DNA methylation assays for the genes analyzed that were already published. Therefore, we developed new pyrosequencing assays by selecting amplicons in promoter CpG-rich areas. For each gene, the PCR-pyrosequencing primer (more than 20 base pairs long) of the highest available quality that was associated with one of the promoters was designed using specialized software (PSQ Assay Design, Biotage, Sweden). The fractions of CpG sites examined by gene were as follows: *TLR-2* (5/49); *F3* (5/78); *NR3C1* (1/7); *ICAM-1* (5/69); *IFN-γ* (2/8); *IL-6* (2/18); *iNOS* (2/8). We did not assay higher proportions of CpG sites due to inherent limitations of the method applied i.e., we excluded PCR amplicons with 350 base pairs or longer, primers that avoided CpGs, and target sequences of 40 base pairs or longer, to optimize PCR and sequencing conditions. Supplementary Table 1 lists the specific CpG positions for DNA methylation that we

measured within specified promoter regions for each gene. We had limited information about the CpGs that were analyzed (e.g., for *NR3C1*), including their functionality or their proximity to transcription factor-binding sites or other important sequences. Because genomic locations were for the hg18 genome build, the majority of the CpGs that we examined were not assayed by the most common methylation assays (i.e. either the 27K or 450K assays) that are available in public datasets.

The degree of methylation was calculated as the percentage of methylated cytosine residues divided by the sum of methylated and unmethylated cytosine residues (%5mC) in each sample. Built-in controls were used to verify bisulfite conversion efficiency. Each sample was tested twice for each marker to improve statistical power and precision. The average of the replicates was used.

Predictor variables. We used data on anxiety and depression measured through the Brief Symptom Inventory (BSI), a self-administered 53-item questionnaire of nine primary psychological symptom dimensions (anxiety, depression, hostility, interpersonal sensitivity, obsessive-compulsive, paranoid ideation, phobic anxiety, psychoticism, somatization) experienced by the respondent over the previous 30 days; the BSI was included as part of the Health and Social Behavior Survey in the NAS starting in 1985.[31,36] Happiness (based on the single item "How happy are you right now?") and life satisfaction (based on the 11-item version of the Life Satisfaction Inventory-A[37]) were also examined as predictor variables. Higher life satisfaction scale scores corresponded to higher self-reported life satisfaction; higher scores on the other scales reflected higher negative psychological symptoms. All psychological measures were analyzed as continuous. Internal consistency reliability (Cronbach's α) values for the anxiety, depression, hostility, and life satisfaction scales were all acceptably high (>0.70).

Covariates. Model covariates consisted of the age at first visit in or after 1999 (years), smoking (pack-years of smoking), education (>high school, ≤high school), history of CHD or stroke prior to

1999, history of diabetes prior to 1999, % basophils, % eosinophils, % lymphocytes, % monocytes, % neutrophils, and plasma folate levels. Previous evidence suggests that leukocyte composition is related to DNA methylation,[38] and that folate is a source of methyl groups and folate depletion leads to lower blood DNA methylation.[39] Because 98% of the sample was White, we did not adjust for race/ethnicity. In sensitivity analyses, we additionally controlled for baseline hypertension (i.e., hypertension prior to 1999) and total serum cholesterol.

Statistical analysis. We first calculated descriptive statistics (mean, range, percentages for psychological factors and covariates, mean percentage methylation for gene-specific promoter methylation) based on study participants with measures of ICAM-1 promoter region methylation, which showed several significant associations.

We then constructed a Pearson correlation coefficient matrix for the psychological factors and a correlation coefficient matrix for the methylation outcomes.

To examine the associations between the psychological factors and the methylation outcomes, we next estimated repeated measures linear mixed models (equivalent to random intercept models) to account for up to four repeated measures, using a first-order autoregressive covariance structure (in which a decreasing correlation of standard errors over time was modeled). The log-likelihood fit statistics for the models indicated better model fits than those for the corresponding models using a compound symmetry covariance structure; unstructured covariance structure models did not converge. Because we assumed a short latency period for methylation changes,[40-43] we modeled each psychological factor as a predictor of gene-specific methylation measured on the same visit (averaged across cytosines in CpG sites within the promoter region, varying from one CpG site for the *NR3C1* gene to five CpG sites for the *F3* gene according to the density of CpG sites in the sequence amplified within the promoter region). In addition, we noted the associations between selected covariates (age, smoking, income/education) and methylation.

For primary associations significant at the 5% level, we further tested for dose-response relationships, by grouping the respective psychological factor into meaningful and/or equally-sized categories where possible. A dose-response relationship would lend support to a casual association.[44] A linear test for trend was performed by converting the categories into an ordinal variable and noting its corresponding p value.

We further examined the associations between psychological factors and serum ICAM-1, to examine whether similar relationships were present as between the psychological factors and ICAM-1 promoter methylation levels (because the latter would be expected to be inversely related to ICAM-1 expression).

Finally, because of the known association between aging and methylation, we repeated the analyses using age^2 as an additional covariate to saturate the model for an age effect and found comparable results (data not shown). Additional sensitivity analyses explored the robustness of the findings after controlling for household income, baseline hypertension, and total serum cholesterol.

All tests were two-tailed with a 5% significance level. All analyses were conducted using SAS Version 9.1 (SAS Institute, Cary, NC).

All participants gave written informed consent. This research was approved by the human subjects committees of the Boston VA Medical Center and the Harvard School of Public Health.

RESULTS

Characteristics of study sample. Table 1 shows descriptive characteristics of the study sample based on 616 men with measures of ICAM-1 promoter region methylation. We present characteristics for this sample because several of the corresponding associations with ICAM-1 methylation were significant among the different gene promoter regions analyzed. The sample had a

mean age of 72.5 years (range 56-100 years) at first visit. Approximately one-third (34.1%) attained no more than high school education and over two-thirds had previously smoked, with an average of 21.8 pack-years of smoking (Table 1). These characteristics were similar to those of the larger cohort of men with visits between 1999 and 2006 including men with missing observations for methylation (n = 1,121 men: mean age 71.7 years, % with less than high school education = 35.9; mean pack-years of smoking = 21.6). After listwise deletion of missing data in respective models, the sizes of analytic samples ranged from 481 to 669 men. Missing gene-specific methylation data ranged from 5.4% (*IFN-γ*) to 23.8% (*iNOS*), due to the presence of assay failures and the lack of sufficient DNA, which disproportionately affected genes that were tested later in the order (i.e., *iNOS*, *ICAM-1*). Missing model covariate data ranged collectively from 3.1% to 3.5%. Missing psychological factor data ranged from 3.7% (happiness) to 10.8% (life satisfaction) in the respective model (Supplementary Table 2). Mean leukocyte methylation levels within promoter regions ranged from 2.2% 5mC (OGG gene) to 84.8% 5mC (IFN-γ gene); none of the distributions was highly skewed (Table 1). Intra-individual changes in leukocyte methylation ranged from 1.4-2.4 times the standard deviation across repeated measures.

Anxiety, depression, and hostility scale scores were significantly positively correlated with one another, and were nearly all significantly inversely correlated with happiness and life satisfaction scores (all $|r|>0.3$ and $p<0.01$; Table 2). By contrast, none of the methylation outcomes were moderately to strongly correlated with one another (all $|r|<0.3$; data not shown), suggesting that these outcomes represented relatively independent events and processes.

Associations between psychological factors and average DNA methylation. Table 3 shows the multivariate-adjusted coefficient estimates from repeated measures models. Negative psychological factors were related to higher average methylation in *ICAM-1* promoter regions (with the associations for anxiety significant at the 0.10 level and for depression significant at the 0.05

level). Happiness was significantly inversely associated with *ICAM-1* promoter methylation. Depression was significantly positively associated and happiness and life satisfaction were significantly inversely associated with average methylation in *F3* promoter regions, respectively. For *TLR-2* promoter methylation, all negative psychological factors showed positive relations (with the association for hostility significant at the 0.10 level) and both positive psychological factors showed inverse relations (with the association for life satisfaction significant at the 0.05 level). For *iNOS* promoter methylation, all negative psychological factors showed inverse relations and both positive psychological factors showed positive relations. However, only the association for life satisfaction was significant at the 0.10 level. For *NR3C1* promoter methylation, depression, hostility, happiness, and life satisfaction all exhibited positive and non-significant associations. Likewise, psychological factors were inconsistently and non-significantly related to higher methylation in the promoter regions for *IFN-γ* and *IL-6*.

For all associations significant at the 0.05 level, we further identified monotonic dose-response relationships, with categories of higher scores of the psychological factors being associated with stronger associations. Tables 4 and 5 show the coefficient estimates across categories as well as the p values from the tests for linear trend across categories; these p values were significant at the 0.05 level for *F3* promoter methylation and at the 0.10 level for *ICAM-1* promoter region methylation, respectively.

In all models, pack-years of smoking significantly predicted higher average methylation levels in the gene-specific promoter regions. Age was non-significantly inversely associated with methylation. Additional adjustment for household income (with lower income being non-significantly positively associated with methylation), baseline hypertension, and total serum cholesterol did not alter the main results (data not shown).

Associations between psychological factors and serum ICAM-1. No psychological factors were associated with serum ICAM-1 levels (for anxiety: β=5.11, p=0.51; other psychological factors exhibited similar associations). ICAM-1 methylation levels and serum ICAM-1 levels were uncorrelated (r = -0.04).

DISCUSSION

In this study of community-dwelling elderly adult men, we found consistent associations between both positive and negative psychological factors with higher average leukocyte DNA methylation in *ICAM-1* promoter regions and in *F3* promoter regions. There was some evidence that hostility was positively associated with *TLR-2* promoter methylation, and that life satisfaction was inversely associated with both *TLR-2* and *iNOS* promoter methylation. We observed less consistent and significant associations between psychological factors and average methylation for promoters of the genes for *NR3C1*, *IFN-γ*, and *IL-6*.

Our main findings were generally robust across multiple Brief Symptom Inventory (BSI) component scales. While this may stem from similarities across component scale measures, results using very different scales (e.g., life satisfaction) were qualitatively consistent. Moreover, smoking has been linked to pro-inflammatory states and atherosclerosis,[45] and the direction of the associations for smoking with hypermethylation of *ICAM-1* promoter regions matched those for negative psychological factors, providing support that the associations were not simply attributable to chance. Our findings were furthermore robust to the adjustment of the presence of CHD, stroke, and diabetes, countering underlying co-morbidities/health selection as alternative explanations for the main findings.

Higher circulating levels of serum *ICAM-1* have been previously independently linked to modest risks of CHD after adjusting for key covariates such as SES.[46-48] Notably, we found no

association between psychological factors and serum *ICAM-1*. Along with the presence of associations between psychological factors and *ICAM-1* promoter methylation, this could be explained by the fact that serum *ICAM-1* is derived from multiple sources (vascular endothelium, macrophages, lymphocytes), consistent with the absence of a correlation between leukocyte *ICAM-1* methylation and serum *ICAM-1*. Past investigations of the Normative Aging Study have likewise found no association between serum *ICAM-1* and *LINE-1* leukocyte methylation levels.[49] Whether methylation of *ICAM-1* in white blood cells predicts serum *ICAM-1* levels derived solely from white blood cells (vs. other sources), and whether this *ICAM-1* independently contributes to higher risks of CHD should be explored in future studies.

Atherosclerosis is a chronic inflammatory process involving the infiltration of leukocytes into the extravascular space, mediated in part by adhesion molecules. Smooth muscle cells participate in this process by expressing adhesion molecules such as vascular cell adhesion molecule-1 (*VCAM-1*) and intercellular adhesion molecule-1 (*ICAM-1*).[50] *ICAM-1* plays a pivotal role in the adhesion of leukocytes to the endothelium.[51-53] Given evidence that psychological factors are risk factors for atherosclerosis,[1] one possible explanation for negative psychological factors being linked to higher *ICAM-1* promoter region methylation in leukocytes is *cellular signaling*, with *ICAM-1* being known to function via signal transduction[54,55] Low binding of leukocyte *ICAM-1* to its cell membrane integrins could trigger a cascade of pro-inflammatory mediators and signal endothelial cells to release *ICAM-1*,[53,56-58] and could thereby stimulate *ICAM-1* leukocyte binding to vascular endothelial cells. Hence, through signaling mechanisms, low leukocyte *ICAM-1* levels could induce leukocyte migration into vascular endothelial tissues. Future biological studies (e.g., animal experiments which manipulate distress or other exposures) should further investigate and test this and other potential pathways.

Depression was positively associated and happiness and life satisfaction were each inversely associated with higher *F3* promoter methylation in leukocytes (which in turn would be linked to reduced leukocyte F3 expression). Some evidence suggests that the major source of *F3* in arterial thrombosis is the vascular wall rather than monocytes,[25] although monocyte *F3* also contributes to inflammation and thrombosis. *F3*, also known as Tissue Factor, has been shown to be involved in cellular signaling and inflammatory pathways.[26,27] Like the hypothesis for *ICAM-1*, low leukocyte *F3* levels via signaling pathways may promote inflammatory states through greater vascular *F3* levels.

Furthermore, hostility was positively associated and life satisfaction was inversely associated with higher *TLR-2* promoter methylation, which would imply lower *TLR-2* expression. These findings appear contrary to the hypothesized role that *TLR-2* plays in atherosclerosis.[28,29] Nonetheless, there is some evidence to suggest that *TLR-2* promoter hypermethylation is present in chronic inflammatory processes such as periodontitis.[30] In addition, it has been suggested that the inflammatory process itself may induce cytosine damage and aberrant methylation patterns, including hypermethylation.[59] Furthermore, the association of negative psychological states such as hostility with decreased expression of TLR-2 may signify suppression of the immune system; this is consistent with observed relationships between stress and immune suppression in other studies.[60]

We found no associations between psychological factors and leukocyte *NR3C1* promoter methylation. Previous studies in humans have yielded conflicting results. For example, an investigation in prenatal women using clinically-administered (Hamilton Rating) scales of anxiety and depression and a self-administered (Edinburgh Postnatal Depression) scale of depression observed associations between higher maternal anxiety and depressive symptom scores and methylation of CpGs within the promoter and exon 1F of the *NR3C1* gene (homologous to the l_7 region of the rat *NR3C1* gene) in maternal blood leukocytes.[12] A study of men and women aged 18-

59 reported correlations between a history of childhood adversity with higher leukocyte *NR3C1* gene promoter methylation, yet found no correlations for anxiety (using the State-Trait Anxiety Inventory) and only limited correlations for depression (using the Inventory for Depressive Symptoms) with GR promoter methylation (at 0 of 13 CpG sites and 2 of 13 CpG sites, respectively).[20] Meanwhile, a recent brain post-mortem study in adults found no hippocampal GR promoter methylation differences between those clinically diagnosed with major depression versus controls.[61]

Strengths of our study include its examination of multiple psychological factors (both positive and negative) and its novel exploration of DNA methylation in promoter regions of multiple genes plausibly involved in chronic immune/inflammatory processes and inflammation-related endothelial dysfunction; and its reliance on a community-based sample which strengthens generalizability of our findings. We further tested for and confirmed linear dose-response relationships, which support the presence of causal associations.

There were several limitations to our study. First, we examined DNA methylation at a subset of CpG sites within a gene promoter region. The inability to assay high proportions given methodological limitations could have led us to the omission of some relevant CpG sites. The analyzed CpGs (selected based on aforementioned methodological limitations) may not necessarily have been good proxies for the rest of the CpGs within the same regions. Second, differences in results from previous studies, particularly for *NR3C1* methylation, might also stem from the measurement of methylation in peripheral blood rather than hippocampal tissue; methylation effects may be tissue specific.[20,62] Third, due to the multiple associations examined, the multiple comparisons problem, whereby multiple comparisons may increase the presence of significant associations by chance, cannot be ruled out. Fourth, while the null associations for methylation in promoter regions of several genes including *NR3C1*, *IFN-γ*, and *IL-6* could reflect the true absence

of associations, they could also possibly be attributed to selection bias due to attrition or missing methylation data, as suggested by demographic (age, education) differences in those analyzed versus the NAS cohort in 1985 when the BSI was first administered. For instance, those with a stronger association between the psychological factors and methylation may have either died or have been lost to follow-up, leading to attenuated and null associations in the analyzed data. With respect to the varying sample sizes between analytic samples for genes examined, the mechanism of missing data due to insufficient DNA and assay failures was plausibly missing completely at random (MCAR), and entirely unrelated to the levels of methylation of a particular sequence of DNA.[32] Under the MCAR mechanism, the listwise deletion method that we applied should be valid.[63] In support of the MCAR assumption being met, we determined that those participants with and without missing methylation data for each gene were generally comparable on demographic characteristics (mean age, distribution of education), mean pack-years of smoking, and mean anxiety and depression scores. Fifth, the NAS cohort does not currently have genome-wide association study (GWAS) data. Hence, we could not specifically evaluate the interplay between genetics and DNA methylation, and further studies are warranted. Sixth, we lacked measures of additional cell subtypes (e.g., B cells, T cells, and natural killer cells, as subtypes of lymphocytes), which may have biased our results through residual confounding. Finally, the presence of null associations may in part be due to the study sample being limited to an elderly, primarily white male population. Effects of psychological factors on DNA methylation may be more salient in other population sub-groups, or at earlier, sensitive time-points over the life-course. Future studies should extend examination of these associations to younger adults, older women, and members of other racial/ethnic groups.

In summary, our study primarily suggests novel relations between positive and negative psychological factors and methylation of ICAM-1 promoter regions and linkages with F3 gene

methylation, and to a lesser extent associations with *TLR-2* promoter methylation. Confirming these findings in other populations and settings may yield a better understanding of the epigenetic mechanisms by which psychological factors influence CHD and other major chronic disease outcomes.

Contributorship Statement

DK, LDK, and JS conceived and designed the study. AB, DS, AS, LT, LC, PV, and JS gathered data. DK performed all data analyses, and drafted the manuscript. DK, LDK, AB, and JS revised the manuscript for important intellectual content.

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Competing Interests

There are no competing interests.

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Data Sharing Statement

Data are from the Normative Aging Study, whose restricted data are available for researchers who meet the criteria.

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Table 1. Descriptive statistics (mean values with ranges in parentheses; percentages) for samples analyzed with respective characteristic and ICAM-1 promoter methylation (n ranging from 538 to 577 men)

Mean age in yrs at first visit in 1999	72.5 (56-100)
% ≤High school	34.1
% White	98.0
% with CHD/stroke/diabetes before 1999	33.3
Smoking in pack-years	21.8 (0-131)
Anxiety	0.20 (0-2.83)
Depression	0.20 (0-3.33)
Hostility	0.21 (0-3.00)
Happiness	7.39 (1-9)
Life satisfaction	7.88 (0-11)
% Basophils	0.56 (0-2)
% Eosinophils	3.24 (0-22)
% Lymphocytes	26.0 (5-90)
% Monocytes	8.76 (0-17)
% Neutrophils	61.65 (3-85)
Plasma folate (ng/mL)	17.41 (3.3-99.3)
DNA methylation in gene promoter regions (%)	
TLR-2	3.1 (0-8.9)
F3	2.3 (0-14.8)
NR3C1	47.0 (14.7-72.8)

ICAM-1	4.4 (1.7-16.1)
IFN-γ	84.4 (30.9-95.7)
IL-6	43.7 (10.3-86.6)
iNOS	69.7 (24.5-87.2)

Table 2. Pearson correlation coefficients between psychological factors.[a]

	Anxiety	Depression	Hostility	Happiness	Life satisfaction
Anxiety	1.00	0.76 (n=611)	0.67 (n=611)	-0.32 (n=612)	-0.31 (n=578)
Depression		1.00	0.63 (n=609)	-0.46 (n=611)	-0.42 (n=577)
Hostility			1.00	-0.30 (n=610)	-0.28 (n=577)
Happiness				1.00	0.58 (n=598)
Life satisfaction					1.00

[a]For men with observations for the pair of psychological factors.
P<0.01 for all correlations.

Table 3. Coefficient estimates (95% CI) for multivariate associations between psychological factors and average methylation in gene promoter regions, from repeated measures models.

	Gene						
	TLR-2	*F3*	*NR3C1*	*ICAM-1*	*IFN-γ*	*IL-6*	*iNOS*
Anxiety	0.07 (-0.17, 0.32) n=558; 833 obs	0.17 (-0.05, 0.40) n=607; 909 obs	-0.42 (-1.54, 0.71) n=581; 924 obs	0.34[b] (-0.03, 0.72) n=548; 831 obs	0.50 (-0.41, 1.40) n=640; 1069 obs	0.36 (-1.75, 2.47) n=636; 1077 obs	-0.82 (-2.28, 0.64) n=499; 729 obs
Depression	0.08 (-0.15, 0.30) n=554; 825 obs	0.34[a] (0.14, 0.55) n=605; 904 obs	0.22 (-0.76, 1.21) n=579; 919 obs	0.38[a] (0.04, 0.72) n=546; 826 obs	0.21 (-0.62, 1.04) n=638; 1064 obs	-0.12 (-2.07, 1.83) n=634; 1071 obs	-0.60 (-1.93, 0.73) n=496; 723 obs
Hostility	0.22[b] (-0.04, 0.49) n=554, 828 obs	0.18 (-0.06, 0.42) n=603; 905 obs	0.20 (-1.00, 1.40) n=578; 921 obs	0.20 (-0.19, 0.60) n=545; 828 obs	0.39 (-0.56, 1.34) n=636; 1066 obs	-0.54 (-2.74, 1.66) n=632; 1074 obs	-0.34 (-1.82, 1.14) n=497; 727 obs
Happiness	-0.02 (-0.09, 0.05) n=582; 867 obs	-0.10[a] (-0.16, -0.04) n=636; 952 obs	0.12 (-0.17, 0.41) n=608; 967 obs	-0.10[a] (-0.22, -0.003) n=577; 871 obs	0.04 (-0.20, 0.28) n=669; 1117 obs	-0.38 (-0.95, 0.19) n=666; 1128 obs	0.07 (-0.33, 0.47) n=523; 760 obs
Life Satisfaction	-0.05[a] (-0.09, -0.01) n=539; 808 obs	-0.06[a] (-0.10, -0.03) n=590; 885 obs	0.09 (-0.09, 0.26) n=563; 895 obs	-0.02 (-0.08, 0.04) n=538; 813 obs	-0.04 (-0.19, 0.10) n=619; 1036 obs	0.15 (-0.18, 0.49) n=615; 1045 obs	0.20[b] (-0.02, 0.43) n=481; 698 obs

Associations between each psychological factor and average levels of methylation across CpG sites within gene promoter regions examined in separate models. All models adjusted for age, smoking status, educational attainment, history of CHD or stroke prior to 1999, history of diabetes prior to 1999, % basophils, % eosinophils, % lymphocytes, % monocytes, % neutrophils, and plasma folate.
[a]P<0.05. [b]P<0.10.

Table 4. Coefficient estimates from repeated measures models for multivariate associations between categorized scale values of depression, happiness, life satisfaction and *F3* promoter methylation (n = 658 men, 988 observations).

		Coefficient Estimate	95% CI	P value
Depression				
	0	-	-	-
	0.01-0.4	-0.13	-0.34, 0.09	0.24
	>0.4	0.33	0.10, 0.56	0.005
				$P_{trend} = 0.03$
Happiness				
	1-4 (unhappy)	-	-	
	5-7	-0.20	-0.54, 0.14	0.24
	8-9 (happy)	-0.51	-0.85, -0.18	0.003
				$P_{trend} <.001$
Life satisfaction				
	0-5	-	-	-
	6-8	-0.28	-0.49, -0.06	0.01
	9-11	-0.40	-0.60, -0.20	<0.001
				$P_{trend} <.001$

F3 methylation values corresponded to the average levels of methylation across CpG sites within the *F3* promoter region.
All models adjusted for age, smoking status, educational attainment, history of CHD or stroke prior to 1999, history of diabetes prior to 1999, % basophils, % eosinophils, % lymphocytes, % monocytes, % neutrophils, and plasma folate.

Table 5. Coefficient estimates from repeated measures models for multivariate associations between categorized scale values of depression and happiness and *ICAM-1* promoter methylation (n = 600 men, 906 observations)

		Coefficient Estimate	95% CI	P value
Depression				
	0	-	-	-
	0.01-0.4	0.19	-0.16, 0.55	0.29
	>0.4	0.30	-0.09, 0.70	0.13
				$P_{trend} = 0.09$
Happiness				
	1-4 (not happy)	-	-	-
	5-7	-0.21	-0.76, 0.34	0.46
	8-9 (happy)	-0.42	-0.97, 0.13	0.13
				$P_{trend} = 0.06$

ICAM-1 methylation values corresponded to the average levels of methylation across CpG sites within the *ICAM-1* promoter region.
All models adjusted for age, smoking status, educational attainment, history of CHD or stroke prior to 1999, history of diabetes prior to 1999, % basophils, % eosinophils, % lymphocytes, % monocytes, % neutrophils, and plasma folate.

Supplementary Table 1. Location of the CpG position and the promoter region for each gene.*

Gene	Chromosome	Promoter		CpG Positions for measured DNA methylation				
		Start	End	Position 1	Position 2	Position 3	Position 4	Position 5
TLR-2	4	154824391	154824991	154824709	154824713	154824715	154824723	154824727
F3	1	94779671	94780502	94779947	94779950	94779956	94779958	94779974
NR3C1	5	142760496	142761097	142760565				
ICAM-1	19	10242017	10242937	10242236	10242225	10242218		
IFN-γ	12	66839561	66840293	66840192	66840186			
Il-6	7	22732791	22733685	22733847	22733841			
iNOS	17	23149861	23150461	23149929	23149936			

*NCBI build 36.1 was used as the reference of the human genome in this study.

Supplementary Table 2. Numbers and percentages of missing men for methylation in the promoter region for each gene, for model covariates, and respective psychological factors (n = 765 men without excluding those with missing values).

Psychological Factor	*TLR-2*	*F3*	*NR3C1*	*ICAM-1*	*IFN-γ*	*IL-6*	*iNOS*
Anxiety	Missing methylation: n=123; 16.1% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=74; 9.7% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=100; 13.1% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=133; 17.4% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=41; 5.4% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=45; 5.9% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)	Missing methylation: n=182; 23.8% Missing covariates: n=26 (3.4%) Missing anxiety: n=58 (7.6%)
Depression	Missing methylation: n=125; 16.3% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=74; 9.7% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=100; 13.1% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=133; 17.4% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=41; 5.4% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=45; 5.9% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)	Missing methylation: n=183; 23.9% Missing covariates: n=27 (3.5%) Missing depression: n=59 (7.7%)
Hostility	Missing methylation: n=123; 16.1%	Missing methylation: n=74; 9.7%	Missing methylation: n=99; 12.9%	Missing methylation: n=132; 17.3%	Missing methylation: n=41; 5.4%	Missing methylation: n=45; 5.9%	Missing methylation: n=180; 23.5%

	Missing covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	Missing covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	Missing covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	Missing covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	Missing covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	Missing covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)	Missing covariates: n=27 (3.5%) Missing hostility: n=61 (8.0%)
Happiness	Missing methylation: n=128; 16.7% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=74; 9.7% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=102; 13.3% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=133; 17.4% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=41; 5.4% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=44; 5.8% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)	Missing methylation: n=187; 24.4% Missing covariates: n=27 (3.5%) Missing happiness: n=28 (3.7%)
Life satisfaction	Missing methylation: n=119; 15.6% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=68; 8.9% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=95; 12.4% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=120; 15.7% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=39; 5.1% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=43; 5.6% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)	Missing methylation: n=177; 23.1% Missing covariates: n=24 (3.1%) Missing life satisfaction: n=83 (10.8%)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	**Comment [DK1]:** Page 1 (Abstract)
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	**Comment [DK2]:** Page 1 (Abstract)
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	**Comment [DK3]:** Pages 3 and 4
Objectives	3	State specific objectives, including any prespecified hypotheses	**Comment [DK4]:** Bottom of page 4
Methods			
Study design	4	Present key elements of study design early in the paper	**Comment [DK5]:** Top of page 5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	**Comment [DK6]:** Page 5
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	**Comment [DK7]:** Page 5
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	**Comment [DK8]:** Pages 5-8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	**Comment [DK9]:** Pages 5-8
Bias	9	Describe any efforts to address potential sources of bias	**Comment [DK10]:** Bottom of page 7, top of page 8
Study size	10	Explain how the study size was arrived at	**Comment [DK11]:** Page 10 and Supplementary Table 2
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	**Comment [DK12]:** Pages 5-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	**Comment [DK13]:** Pages 8=9
		(*b*) Describe any methods used to examine subgroups and interactions	**Comment [DK14]:** Tests for analyses not performed.
		(*c*) Explain how missing data were addressed	**Comment [DK15]:** Top of page 10
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	**Comment [DK16]:** Not addressed but attrition noted as study limitation on page 16.
		(*e*) Describe any sensitivity analyses	**Comment [DK17]:** Page 9 - NEW SENTENCE HAS BEEN ADDED HERE.

Continued on next page

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	**Comment [DK18]:** Further details of participation/enrollment of original study participants are given in citation at top of page 5.
		(b) Give reasons for non-participation at each stage	**Comment [DK19]:** Further details of participation/enrollment of original study participants are given in citation at top of page 5.
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	**Comment [DK20]:** Page 10 and Table 1
		(b) Indicate number of participants with missing data for each variable of interest	**Comment [DK21]:** Page 10 and Supplemental Table 2
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	**Comment [DK22]:** Follow up from 1999 to 2006 noted on page 5.
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	**Comment [DK23]:** Page 10 (# events same as sample sizes noted on this page as outcomes were analyzed as continuous)
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	**Comment [DK24]:** Page 8, 10-12, Tables 3-5 (pages 29-31)
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	**Comment [DK25]:** Top of page 12
Discussion			
Key results	18	Summarise key results with reference to study objectives	**Comment [DK26]:** Bottom of page 12, top of page 13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	**Comment [DK27]:** Bottom of page 15, all of page 16
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	**Comment [DK28]:** Pages 12-16
Generalisability	21	Discuss the generalisability (external validity) of the study results	**Comment [DK29]:** Bottom of page 16
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	**Comment [DK30]:** Page 18

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.