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Effects of Temperature and Relative Humidity on DNA Methylation

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Abstract

Background—Previous studies have found relationships between DNA methylation and various environmental contaminant exposures. Associations with weather have not been examined. Because temperature and humidity are related to mortality even on non-extreme days, we hypothesized that temperature and relative humidity may affect methylation.

Methods—We repeatedly measured methylation on long interspersed nuclear elements (*LINE-1*), *Alu*, and 9 candidate genes in blood samples from 777 elderly men participating in the normative aging Study (1999–2009). We assessed whether ambient temperature and relative humidity are related to methylation on *LINE-1* and *Alu*, as well as on genes controlling coagulation, inflammation, cortisol, DNA repair, and metabolic pathway. We examined intermediate-term associations of temperature, relative humidity, and their interaction with methylation, using distributed lag models.

Results—Temperature or relative humidity levels were associated with methylation on tissue factor (*F3*), intercellular adhesion molecule 1 (*ICAM-1*), toll-like receptor 2 (*TRL-2*), carnitine O-acetyltransferase (*CRAT*), interferon gamma (*IFN-γ*), inducible nitric oxide synthase (*iNOS*), and glucocorticoid receptor, *LINE-1*, and *Alu*. For instance, a 5°c increase in 3-week average temperature in *ICAM-1* methylation was associated with a 9% increase (95% confidence interval:

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3% to 15%), whereas a 10% increase in 3-week average relative humidity was associated with a 5% decrease (−8% to −1%). The relative humidity association with *ICAM-1* methylation was stronger on hot days than mild days.

Conclusions—DNA methylation in blood cells may reflect biological effects of temperature and relative humidity. Temperature and relative humidity may also interact to produce stronger effects.

High- and low-ambient temperatures, extreme or not, have been associated with increased risk for cardiovascular mortality,[1,2] especially in the elderly.[3] Biological mechanisms of the adverse effects of temperature have not been fully understood. Temperature changes may induce inflammatory responses,[4-7] change blood viscosity,[7,8] change heart-rate variability,[9] affect blood pressure,[10,11] cause myocyte injury,[6] or modify cholesterol levels.[8,12] The biological pathways for the possible effects of humidity on mortality have received less attention.[1,2] Koken et al[1] related dew-point temperature to coronary atherosclerosis and congestive heart failure. Other studies have focused on the adverse effects of apparent temperature; however, their results were inconsistent.[13-17] In these studies, apparent temperature was constructed to reflect the physiological experience of exposure to both temperature and humidity, which may capture health effects better than using temperature alone. To the best of our knowledge, a health effect of humidity independent of temperature has not been demonstrated.

Recent research has linked epigenetics to cardiovascular disease.[18,19] We hypothesized that epigenetics may also play a role in the adverse effects of weather on the cardiovascular system. The most studied epigenetic mechanism is DNA methylation, which refers to the addition of a methyl group on cytosine bases. Defects in the methylation machinery resulting in hypomethylation or hypermethylation, depending on the gene it regulates, can have devastating consequences including serious diseases.[20] DNA methylation can act as a switch in gene expression because its distribution is usually bimodal, with regions either highly methylated or unmethylated.[21] Moreover, although protein levels can change within an hour, epigenetic marks are more stable and possibly a better choice for examining associations with environmental exposures averaged over longer periods such as weeks.[22,23] Previous studies have observed changes in DNA methylation also occurred after exposure to air pollution.[24,25] In addition, Madrigano et al[26] demonstrated that gene-specific methylation changes with time. Increased age was related to tissue factor (*F3*), interferon gamma (*IFN-γ*) carnitine O-acetyltransferase (*CRAT*), and 8-oxoguanosine DNA glycosylase-1 (*OGG1*) hypermethylation, and inducible nitric oxide synthase (*iNOS)*, toll-like receptor 2 (*TLR-2*), and glucocorticoid receptor (*GCR*) hypomethylation.

Epigenetic control also plays an important role in inflammatory gene expression.[27] Therefore, we hypothesized that temperature and relative humidity (independently or synergistically) may change methylation on interspersed nuclear elements (*LINE-1*), *Alu*, and 9 candidate genes related to coagulation, inflammation, cortisol, DNA repair, and metabolic pathway. Because the elderly are more susceptible to the adverse effects of temperature,[3] we focused our investigation on an elderly population having repeated measurements of DNA methylation.

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METHODS

Study Population

The study population consisted of participants from the Normative Aging Study, an investigation of aging community-dwelling men from the Greater Boston area.[27] Our analyses included 777 participants who visited the examination site every 3 to 5 years to undergo physical examinations (n = 1798 observations). We started to measure DNA methylation on blood samples collected after 1999. We restricted our analyses to participants with C-reactive protein concentrations less than 10 mg/L so that the results are not confounded by infection state. This study was approved by the review board of all participating institutions.

Weather and Air Pollution Assessments

We obtained measurements of dew-point and ambient temperatures, relative humidity, and barometric pressure from the Boston Logan Airport weather station located 8 km from the study center. Because study participants lived throughout the metropolitan area, we assumed that the monitored temperature and humidity can serve as surrogates of their exposures. We calculated apparent temperature, vapor pressure, and absolute humidity using the following formulae:

$$\begin{aligned}&\text{Apparent temperature}\,(^{\circ}\text{C})\\&=-2.653+0.994\times\text{Ambient temperature}\,(^{\circ}\text{C})+0.0153\times\text{Dew point temperature}(^{\circ}\text{C})^{2}\end{aligned}$$

$$\begin{aligned}&\text{Water vapor pressure}\,(Pa)\\&=611\times 10^{(7.5^{*}\text{dew point temperature}(^{\circ}\text{C}))/(237.7+\text{dew point temperature}(^{\circ}\text{C}))}\end{aligned}$$

$$\begin{aligned}&\text{Absolute humidity}\left(kg/\text{m}^{3}\right)\\&=\left[\left(\text{Water vapor pressure}\,(Pa)\right)\right]/\left[\left(273.15+\text{Ambient temperature}\,(^{\circ}\text{C})\right)\times 461.5\right]\end{aligned}$$

In this cohort, Halonen et al[28] found intermediate-term associations between temperature averaged up to 1 month and C-reactive protein, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Therefore, we focused our analyses on a similar intermediate-term exposure window and considered averages of temperature and relative humidity over 1–3 weeks preceding each participant's visit.

We monitored fine particles concentrations at the Harvard supersite located in downtown Boston and approximately 1 km from the examination center. $PM_{2.5}$ concentrations were measured with a Tapered Element Oscillation Microbalance (Model 1400a, Rupprecht and Pastaschnick, East Greenbush, NY).

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Epigenetics Assessment

We collected participant's blood at every visit and isolated bisulfite-treated DNA with the Wizard DNA Clean-Up System (Promega, Madison, WI). We assessed DNA methylation using highly quantitative analysis based on polymerase chain reaction pyrosequencing.[29] We chose to measure methylation on genes that are expressed in blood leukocytes. We measured *F3*, *ICAM-1*, *TLR-2*, *CRAT*, *OGG1*, *IFN-γ*, *GCR*, and *iNOS* methylation at 1–5 CpG positions within each gene's promoter region and calculated the mean values of the position-specific measurements (eAppendix 1). Interleukin-6 (*IL-6*) methylation levels were quantified outside the gene's promoter region. The assay measuring gene-specific methylation was developed by locating the promoters using genomatix Software (Genomatix Software Inc, Ann Arbor, MI). The degree of methylation was expressed as a percentage of methylated cytosines over the sum of methylated and unmethylated cytosines at position 5 (%5mC).

Statistical Methods

Assumptions—Because we had repeated measures of methylation for 71% of the participants, we fit generalized mixed-effects models with random intercepts to investigate whether levels of temperature and relative humidity weekly averaged over the 3-week period before the *j*th visit of the *i*th participant were associated with mean methylation of the *i*th participant assessed at the *j*th visit (Y_{ij}). Because *F3*, *ICAM-1*, *TLR-2*, *CRAT*, and *OGG1* methylation had a point mass at zero and their residuals' distribution showed important deviation from a normal density (right skewness), we assumed a tweedie distribution for methylation (eAppendix 2) with a log-link and reported multiplicative effects. For *IFN-γ*, *IL-6*, *iNOS*, *GCR*, *LINE-1*, and *Alu* methylation, we assumed Gaussian distributions for the regression residuals and present our results on the additive scale.

We adjusted for potential confounders (vector $\mathbf{C_1}$) such as: batch of methylation measurement, season (winter/spring-fall/summer), and seasonal sine and cosine with a 365-day cycle. When we modeled the date-methylation relationship using a penalized spline, we obtained a similar seasonal cycle. Therefore, we chose to use the sine and cosine terms to estimate both the amplitude and the phase of the seasonal cycle with only 2 degrees of freedom. We also controlled for the following risk factors of DNA methylation (vector $\mathbf{C_2}$): age, race, diabetes, body mass index, smoking status, statin use, as well as percentages of neutrophil, lymphocyte, monocytes, and basophils in differential blood count (because the 5-methylcytosine distribution in the genome of differentiated somatic cells varies by cell type). We included $\mathbf{C_2}$ in the models for statistical efficiency and to block any potential backdoor path through unmeasured variable *U* that would be a common cause of the exposures of interest and $\mathbf{C_2}$.[30] We thus assumed no unmeasured confounding between the exposures of interest and DNA methylation, given the random intercepts and the $\mathbf{C_1}$ and $\mathbf{C_2}$ vectors.

Moreover, we assumed the missing mechanisms to be at random conditional on the covariates. We also assumed that secondary air pollutants may play the role of intermediate variables between temperature and methylation rather than being confounding variables. Therefore, we chose not to include any air pollutant in our main regression models. We checked this assumption in a secondary analysis in which we controlled for $PM_{2.5}$

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concentrations. We used akaike's information criterion (AIC) to decide whether an alternative model including only apparent temperature instead of both temperature and relative humidity was more appropriate. AIC favored the model including both temperature and relative humidity.

We checked for nonlinear dose-response relationships of temperature and relative humidity, with methylation using generalized additive models and cubic splines. We found no deviation from linear dose-response relationships with respect to methylation. We explored the nature of the temperature and relative humidity associations with methylation over time by fitting distributed lag linear models (lags 0–20 days), and we examined how the relationship between lagged exposures and methylation outcomes changes across lags. This methodology, previously developed for the analysis of time-series data,[31] is extended here in the context of individual longitudinal data. We chose natural splines with 3 degrees of freedom to model the nonlinear shape of the distributed lag. Because the temperature and relative humidity relationships with methylation varied over the exposure lags, we calculated from the distributed lag model the cumulative temperature and relative humidity effects over 1-week periods (lags 0–6, 7–13, and 14–20 days) preceding the j^{th} visit of the i^{th} participant, as well as the cumulative effects over the entire 3-week period.

Main Regression Models

The distributed lag model for *F3*, *ICAM-1*, *TLR-2*, *CRAT*, and *OGG1* methylation (multiplicative scale) is as follows:

$$\begin{aligned} logE\left(\mathrm{Y}_{ij}\right) = \quad & \left(\gamma_0 + \mathrm{u_i}\right) + \mathrm{f}_1\left(\gamma_1, \mathrm{Temperature}_{ij}\right) \\ & + \mathrm{f}_2\left(\gamma_2, \mathrm{relativehumidity}_{ij}\right) \\ & + \Sigma_{\mathrm{k}}\gamma_{4\mathrm{k}}\mathrm{C}_{1kij} + \Sigma_{\mathrm{k}}\gamma_{5\mathrm{k}}\mathrm{C}_{2kij} \end{aligned}$$

with Y_{ij}~Tweedie and $\mathrm{u_i}$~$\mathrm{N}(0,\sigma_u^2)$.

The distributed lag model for *IFN-γ*, *IL-6*, *iNOS*, *GCR*, *LINE-1*, and *Alu* methylation (additive scale) is as follows:

$$\begin{aligned} \mathrm{Y}_{ij} = \quad & \left(\gamma_0 + \mathrm{u_i}\right) + \mathrm{f}_1\left(\gamma_1, \mathrm{Temperature}_{ij}\right) \\ & + \mathrm{f}_2\left(\gamma_2, \mathrm{Relativehumidity}_{ij}\right) \\ & + \Sigma_{\mathrm{k}}\gamma_{4\mathrm{k}}\mathrm{C}_{1kij} + \Sigma_{\mathrm{k}}\gamma_{5\mathrm{k}}\mathrm{C}_{2kij} + \varepsilon_{ij} \end{aligned}$$

with ε_{ij}~$\mathrm{N}(0,\sigma^2)$ and $\mathrm{u_i}$~$\mathrm{N}(0,\sigma_u^2)$

In both of the models, f_1 and f_2 represent the distributed lag functions with sets of coefficients γ_1 and γ_2 constrained by a natural spline (with 3 degrees of freedom) that correspond to the temperature and relative humidity effects at lags 0–20 days. $\boldsymbol{C_1}$ and $\boldsymbol{C_2}$ correspond to sets of variables for which we adjusted.

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Temperature and Humidity Interaction

Focusing only on genes associated with both temperature and relative humidity, we examined whether these 2 weather variables interact to modify methylation. We used a 2-covariate penalized thin plate spline to investigate the combined effect of 3-week average temperature and relative humidity on methylation. We obtained 3-dimensional (3D) perspective plots and examined the shapes of the surface. A plane would suggest no interaction between the 2 variables, whereas a deformed surface would suggest an interaction. In addition to a 3D-perspective plot, we examined interactions by estimating the association between relative humidity and the outcome for 3 temperature levels (determined with the 25th and 75th percentiles of the temperature distribution).

Sensitivity Analyses

Because we chose to model the distributed lag functions with natural splines (with 3 degrees of freedom), we also considered another choice of distributed lag functions that assumes constant lag effect within weeks. Note that this assumption is equivalent to fitting a model that simultaneously includes 4 consecutive weekly exposure moving averages.

For simplicity and to limit the number of tests, our additional secondary analyses examined only the cumulative exposure effect over the entire 3-week period. We adjusted for $PM_{2.5}$ concentrations and barometric pressure to evaluate the role of temperature and humidity in a more complex weather setting. We conducted some multiple testing corrections using Bonferroni adjustment based on 66 tests (n_{tests}= 2 exposures × 11 outcomes × 3 lag times).

RESULTS

Descriptive Statistics

Demographic characteristics of the study participants (median age 72 years) varied according to their number of visits (Table 1 and eAppendix 3). Boston's climate is continental, with direct influences from the ocean. Summers are mostly warm and humid, and winters are usually cold and dry. Temperature and relative humidity distributions showed variability among participants (eAppendix 4). The Pearson correlation coefficient between temperature and absolute humidity was 0.96, and between temperature and relative humidity 0.22 (eAppendix 5). The distributions of gene-specific methylation varied by gene: for instance, the distribution for *IFN-γ* methylation was wider than for *TLR-2* methylation (eAppendices 6a and 6b). The distributions of gene-specific methylation were concentrated around low or high values, except for *IL-6* and *GCR* methylation.

Temperature and Relative Humidity Effects

Temperature (eAppendix 7) and relative humidity (eAppendix 8) were associated with methylation on *F3, ICAM-1, TRL-2, CRAT, IFN-γ, iNOS, GCR, LINE-1*, and *Alu*. For instance, a 5°c increase in temperature (1st week) was related to a 6% decrease in *F3* methylation (95% confidence interval [CI] = −11% to −1%) and a 10% increase in relative humidity (3rd week) was associated with a 0.25%5mC decrease in *IFN-γ* (−0.48 to −0.01). Temperature was not related to *OGG1, IFN-γ, IL-6, iNOS*, and *GCR* methylation, and relative humidity was not associated with *TLR-2, IL-6*, and *OGG1* methylation. The

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distributed lag models indicated different time windows for the associations of temperature and relative humidity with methylation, depending on the genes. For instance, although the temperature association with *F3* methylation was observed during the first week of exposure, the signals on *ICAM-1* were stronger after 3 weeks of exposure.

The associations between temperature and relative humidity exposures over the 3-week period preceding each medical visit and methylation are summarized in Table 2. For example, a 5°c increase in temperature and a 10% increase in relative humidity (3 week) were associated with an 9% increase (95% CI = 3% to 15%) and a 5% decrease (−8% to −1%) in *ICAM-1* methylation, respectively.

Interactions Between Temperature and Relative Humidity

We investigated whether exposures to temperature and relative humidity (3 week) interact to modify levels of *ICAM-1* and *LINE-1* methylation. After fitting a 2-dimensional smoothing of temperature and relative humidity on *ICAM-1* methylation, we obtained a 3D surface that indicated an interaction between temperature and relative humidity during humid and hot days (Figure 1). The relative humidity association with *ICAM-1* methylation was stronger during hot days (Figure 2). We did not find any evidence of an interaction between temperature and relative humidity that modified *LINE-1* methylation.

Sensitivity Analyses

We found similar results when we assumed a distributed lag model with constant lag effects within weeks for temperature and relative humidity versus distributed lag model constrained by natural splines with 3 degrees of freedom (eAppendices 7 and 8).

When we added barometric pressure to our models, the results did not change (Figure 3 and eappendix 9a and 9b for numerical values). We also adjusted for $PM_{2.5}$ in our regression model, with few changes in our primary findings (Figure 3 and eAppendix 9a and 9b for numerical values); except for the temperature estimates on *iNOS* and *GCR* methylation, which became significant, and for the temperature signal on *LINE-1* methylation, which disappeared. After controlling for $PM_{2.5}$, a 5°c increase in temperature (3 week) was associated with an increase of 1.68%5mc in *iNOS* methylation (95% CI = 0.53 to 2.83) and of 1.19%5mc in *GCR* methylation (0.20–2.19). After adjusting for multiple comparisons using the Bonferroni correction, *P* values were 0.04 for the association of relative humidity with *LINE-1* methylation, 0.03 for the association with *Alu* methylation.

DISCUSSION

Our results suggest associations of ambient temperature and relative humidity with DNA methylation on *LINE-1*, *Alu*, and genes related to coagulation, inflammation, cortisol, and metabolic pathway. Our findings support previous studies showing an influence of ambient temperature on biomarkers of inflammation[4,5,28] and heart failure,[6] blood viscosity,[7] heart rate variability,[9] blood pressure,[10,11] and cholesterol.[8,12] A correlation between temperature and methylation on repetitive elements has been reported in a previous study focusing on the association of air pollution with *LINE-1* and *Alu* methylation.[24] Associations of temperature and relative humidity exposures with methylation have not been previously examined.

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A temperature decrease was associated with *ICAM-1* hypomethylation. *ICAM-1* encodes a cell surface glycoprotein that is overexpressed during inflammatory responses. Temperature decrease has previously been related to higher C-reactive protein levels,[5,6,28] a general inflammatory marker, as well as to ICAM-1 and VCAM-1.[28] In our data, a 1%5mc decrease in *ICAM-1* methylation was associated with 0.7% increase in ICAM-1 protein level (95% CI = 0.0 to 1.4). The negative slope we found and the results observed by Zhang et al[21] suggest that *ICAM-1* hypomethylation is related to *ICAM-1* gene desilencing and thus ICAM-1 protein overexpression. Therefore, low levels of *ICAM-1* methylation may be responsible for increased ICAM-1 expression in response to cold temperature.

Similarly, a decrease in temperature was associated with *CRAT* hypermethylation, as well as *GCR* and *iNOS* hypomethylation (after adjustment for $PM_{2.5}$). CRAT is an important enzyme for the cellular cycle and participates in the metabolic pathway in peroxisomes, mitochondria, and endoplasmic reticulum. Changes in CRAT have been related to increased risk of cardiovascular outcomes.[32] in the same cohort, aging was also associated with *CRAT* hypermethylation.[26] *CRAT* hypermethylation may consist of a cellular metabolic response because of decreased temperatures and may be a mechanism that explains the relationship between cold temperatures and cardiovascular-related death. *GCR* is a receptor to which glucocorticoids, such as cortisol, bind. An animal study has related cold weather conditions to increased cortisol secretion in sheep.[33] Therefore, cold temperature exposure may induce *GCR* hypomethylation, which in turn regulates the binding of released cortisol.

iNOS is an enzyme that generates nitric oxide (NO) from the amino acid l-arginine. NO plays an important role in blood pressure regulation, host defense mechanisms, and inflammation.[34] Oxidative-stress-related inflammatory response has also been associated with nuclear factor kappa B (NF-κB) and inOS activity in mice.[35] In an oxidative environment in which inOS is usually formed, NO reacts with superoxide (O_2^-) leading to peroxynitrite ($ONOO^-$) production and thus cell toxicity. When playing a role in host immunity, iNOS participates in eliminating foreign compounds such as reactive oxidative species. Madrigano et al[25] demonstrated that air pollution exposure, which also involves oxidative stress reactions, was associated with *iNOS* hypomethylation in the same elderly cohort. Cold weather conditions may lead to the formation of reactive oxidative species and *iNOS* hypomethylation, enhancing iNOS protein expression.

Temperature increases were associated with *TLR-2* hypomethylation. *TLR-2* is a gene coding a protein that is involved in the activation of innate immunity. TLR-2 and IL-6 may be interrelated, as suggested by a study that found greater effects of air pollution on IL-6 for wildtype mice compared with knockout mice for *TLR-2*.[36] Higher IL-6 levels have also been related to increased C-reactive protein levels,[37] which is also an important player in immune responses as an early defense system against infections. *TLR-2* hypomethylation may activate *TLR-2* gene expression and induce biologic processes enhancing IL-6 and C-reactive protein after exposure to warm temperatures. Although our results do not prove that these methylation pathways are the primary reasons for higher C-reactive protein with higher temperatures reported in previous studies,[4,6,7] they do suggest a plausible role in inflammatory responses because of high temperature.

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We also observed associations between relative humidity and methylation. The lower AIC for the models with both temperature and relative humidity indicates that apparent temperature does not capture the weather relationship with methylation as well as the 2 covariates, suggesting that relative humidity may affect methylation independently of temperature. An increase in relative humidity (1 week) was associated with *ICAM-1* hypomethylation and was suggestively related to *F3* hypomethylation. Although we have already mentioned that ICAM-1 levels are high during inflammatory responses, it is important that tissue factor upregulation and increased fibrin production were observed in hypercoagulable and inflammatory states.[38] Proinflammatory responses are also accompanied by increased tissue factor expression and fibrinogen.[38] Humid weather conditions may lead to *F3* and *ICAM-1* hypomethylation that would increase tissue factor and ICAM-1 expression, respectively. We also observed a negative association between relative humidity (3rd week) and *IFN-γ* methylation. IFN-γ is a cytokine that plays an important role in innate and adaptive immune responses against the intrusion of foreign compounds.[39] Days with high relative humidity could cause changes in *IFN-γ* methylation that would, in turn, regulate the production of the IFN-γ protein. Previous studies have investigated the association between humidity and inflammation.[1,2]

Relative humidity was also associated with *LINE-1* hypomethylation and *Alu* hypermethylation. Similar changes in repetitive element methylation in response to air pollution exposure have been reported in the same cohort.[24] Changes in *LINE-1* and *Alu* methylation have been related to cardiovascular outcomes[29] and could also be potential molecular mechanisms linking weather to cardiovascular mortality.

Our work provides evidence of a potential health effect of relative humidity independent of temperature. Some previous studies examined humidity using dew-point temperature or apparent temperature (which depends on dew-point temperature).[1,13,14,17] Koken et al[1] suggested that humidity has an impact on health and disease, especially on cardiovascular disease. A dew-point temperature increase (from the 25th to the 75th percentile) was related to a 9% increase in risk of hospitalization for coronary atherosclerosis and a 16% increase in congestive heart failure. During cold periods, Wichmann et al[15] observed associations between maximum apparent temperature and admissions for respiratory and cardiovascular diseases only for some susceptible groups, such as the elderly and men. Increases in maximum apparent temperature during the colder months have also been related to a decrease in admissions for acute myocardial infarction.[17] In addition, apparent temperature has been associated with respiratory and circulatory deaths in an elderly population in Vancouver.[14]

The different time windows observed for the associations of temperature and of relative humidity on DNA methylation suggest that molecular responses to temperature and relative humidity may happen either independently from each other or sequentially. Further work is required to explore this (and perhaps other hypotheses) for the difference in time windows. Another interesting finding was the interaction between temperature and relative humidity, which gave rise to stronger decreases in *ICAM-1* methylation during hot and humid days, suggesting a potential overexpression of the ICAM-1 protein after those episodes. Because high ICAM-1 levels have been related to increased risk of cardiovascular events,[40] this

synergistic effect may contribute to cardiovascular morbidity and mortality in the elderly during hot and humid days.

Limitations and Strengths

Our analysis is limited to methylation on 9 genes and 2 repetitive elements. Methylation on other genes and histone modifications might be other important variables to evaluate. We chose 9 genes based on their expression in leukocyte. Because blood is a heterogeneous tissue, it has many leukocytes types and subtypes. Even though we control for the percentages of neutrophils, lymphocytes, monocytes, and basophils in the blood count, there may be some residual confounding. However, when we regressed temperature and relative humidity on percentages of neutrophils, lymphocytes, monocytes, and basophils in blood count in our data, we found no associations. Confounding by cell-type variation is therefore unlikely to explain our findings. We presented results averaged over 1-week to 3-week periods and chose a specific distributed lag function. First, we expect lag-specific estimates to contain more measurement error than estimates using averages. Indeed, when averaging exposure measurements having random noise, some measurement error may be averaged out. Secondly, the distributed lag function we chose may be mis-specified and therefore would bias the estimates. However, because we obtained similar estimates using 2 distributed lag functions, this issue is unlikely to explain our positive results. Not all of our findings were robust to Bonferroni correction. This adjustment is very conservative and assumes independence among the tests, which is unlikely in this study.

Our approach has several strengths. The prospective study design with repeated measures of methylation permitted us to perform a well-powered analysis. High-precision pyrosequencing yields more accurate methylation results than are available from array data. Our findings suggest some independent temperature and humidity relationships with methylation. To our knowledge, these associations have not been reported previously. Furthermore, we fitted distributed lag models and could identify an exposure time window for the temperature and humidity associations with gene-specific and repetitive elements methylation. Our sensitivity analysis indicates that our findings were mostly not the result of confounding by barometric pressure and $PM_{2.5}$ concentrations. We checked for model misspecification by permitting the dose-response relationship between exposures and methylation to be nonlinear and by allowing temperature and humidity to interact. We expect temperature and relative humidity conditions at participants' homes to correlate with conditions at logan airport. The correlation between daily temperatures at logan airport, which exhibits coastal influence, and Worcester Airport, located inland and 80 km away, was 0.95 indicating that weather conditions measured at Logan Airport are reasonable surrogates for residents in Eastern Massachusetts. Previous studies have suggested that temperature changes are associated with relevant cardiovascular events, such as changes in blood viscosity, blood pressure, heart rate variability, and cholesterol. Our study highlights DNA methylation as a biological mechanism that could mediate the health effects of temperature and humidity.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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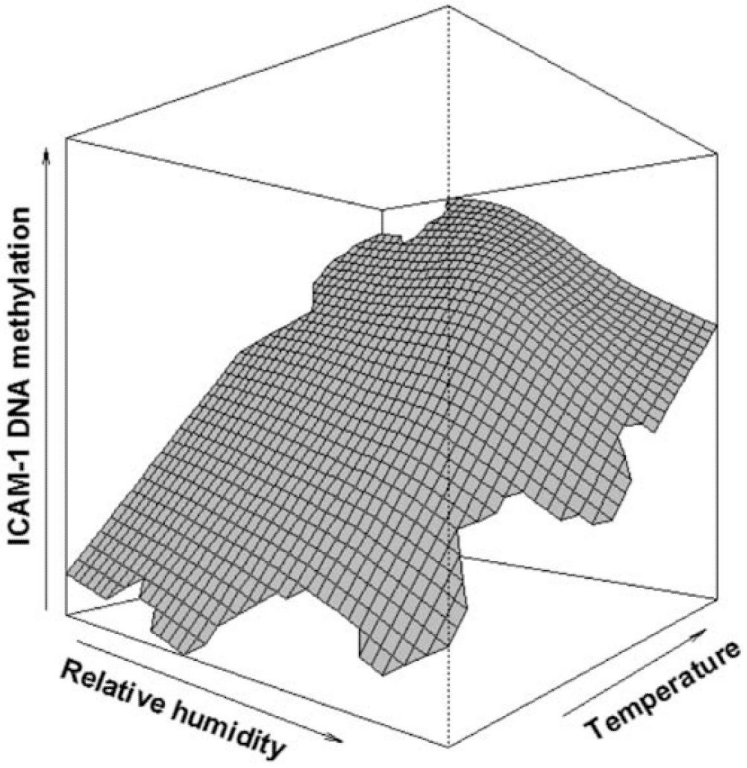

FIGURE 1. Three-dimensional plot obtained after fitting a 2-covariate penalized thin plate spline of temperature and relative humidity on *ICAM-1* methylation.

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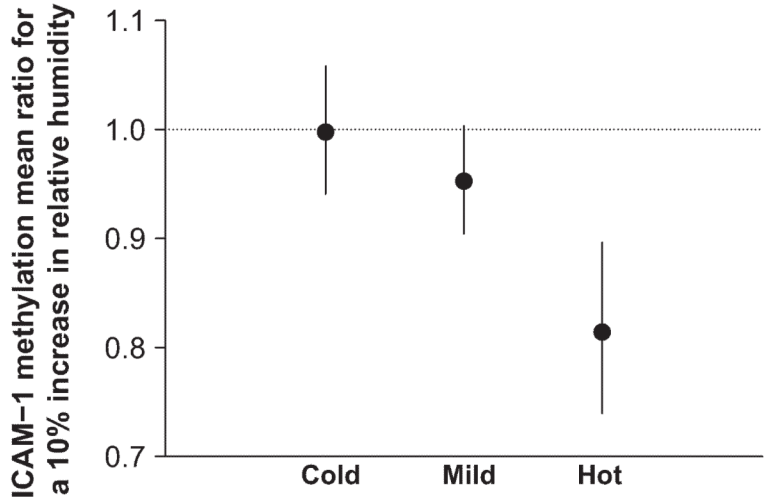

FIGURE 2. Associations between a 10% increase in relative humidity (over a 3-week period) and *ICAM-1* methylation, on cold, mild, and hot days (temperatures below the 25th percentile, between the 25th and 75th percentiles, and above the 75th percentile of the distribution).
Vertical bars indicate 95% CI.

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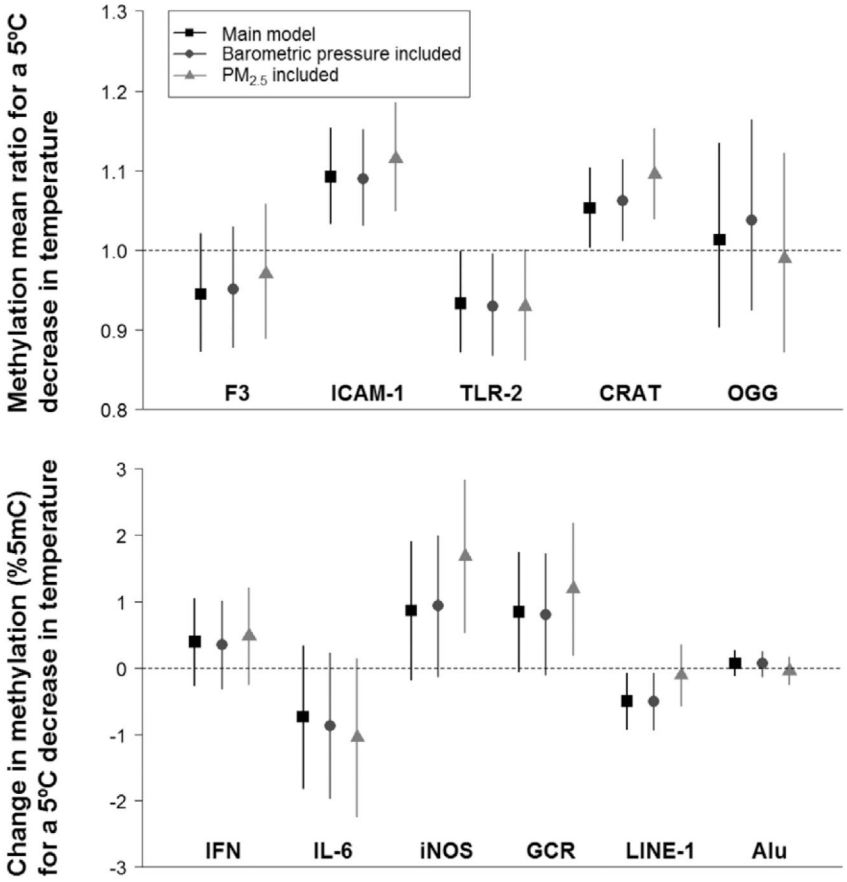

FIGURE 3. Associations between temperature and methylation on specific genes, *LINE-1*, and *Alu*, controlling for barometric pressure and $PM_{2.5}$ concentrations (sensitivity analysis).
Vertical bars indicate 95% CI.

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TABLE 1

Demographic Characteristics of the Normative Aging Study Participants by Number of Visits

	Age (Years) Percentiles			Obesity	Statin User	Diabetic	Smoking		
	5th	50th	95th	%	%	%	Never (%)	Former (%)	Current (%)
Baseline (n = 777)	62	72	84	27	36	14	29	67	4
Among participants having 1 visit (n_1 = 221)									
Visit 1	64	76	88	30	40	18	26	70	4
Among participants having 2 visits (n_2 = 217)									
Visit 1	60	73	83	28	35	16	26	69	5
Visit 2	66	77	86	27	54	19	26	70	4
Among participants having 3 visits (n_3 = 216)									
Visit 1	62	71	82	25	36	9	29	68	3
Visit 2	66	74	86	26	52	13	28	69	3
Visit 3	69	78	89	25	62	17	27	71	2
Among participants having 4 visits (n_4 = 120)									
Visit 1	60	69	77	22	29	10	38	58	4
Visit 2	63	72	81	22	42	11	38	58	4
Visit 3	66	75	84	18	59	16	38	59	3
Visit 4	70	78	87	17	65	18	38	60	2
Among participants having 5 visits (n_5 = 3)									
Visit 1	62	66	66	33	33	0	33	67	0
Visit 2	65	68	70	33	33	0	33	67	0
Visit 3	68	70	72	33	0	0	33	67	0
Visit 4	71	73	74	33	0	0	33	67	0
Visit 5	73	76	77	33	33	0	33	7	0

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TABLE 2

Associations with DNA Methylation of Temperature and of Relative Humidity Over the 3-Week Period Preceding Medical Examination

	Methylation mean ratio for a Δ* increase in temperature and relative humidity (95% CI)				
	F3	***ICAM-1***	***TLR-2***	***CRAT***	***OGGI***
Temperature	0.945 (0.874–1.021)	1.092 (1.034–1.154)	0.933 (0.872–0.999)	1.053 (1.004–1.104)	1.013 (0.904–1.134)
Relative humidity	0.967 (0.921–1.015)	0.952 (0.920–0.985)	0.978 (0.938–1.020)	0.966 (0.920–1.014)	0.971 (0.903–1.043)

	Change in methylation (% 5mC) for a Δ* increase in temperature and relative humidity (95% CI)					
	IFN-γ	***IL-6***	***iNOS***	***GCR***	***LINE-1***	***Alu***
Temperature	0.396 (−0.256 to 1.048)	−0.736 (−1.810 to 0.338)	0.863 (−0.174 to 1.900)	0.845 (−0.053 to 1.743)	−0.497 (−0.915 to −0.080)	0.074 (−0.114 to 0.262)
Relative humidity	−0.289 (−0.684 to 0.106)	0.390 (−0.264 to 1.043)	0.913 (0.253 to 1.572)	0.328 (−0.222 to 0.877)	−0.464 (−0.719 to −0.210)	0.199 (0.083 to 0.314)

Δ* corresponds to increments of 5°C and 10% for temperature and relative humidity, respectively.

Variables included in the models: f_1(temperature), f_2(relative humidity), age, body mass index, smoking status, diabetes status, statin use, % neutrophils in blood count, % lymphocytes in blood count, % monocytes in blood count, % basophils in blood count, seasonal sine and cosine, season, and batch.

f_1 (temperature) and f_2 (relative humidity) represent the distributed lag functions with sets of coefficients constrained by a natural spline (with 3 degrees of freedom) that correspond to the temperature and relative humidity effects at lags 0 and 20.