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Distributional changes in gene-specific methylation associated with temperature

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Abstract

Temperature has been related to mean differences in DNA methylation. However, heterogeneity in these associations may exist across the distribution of methylation outcomes. This study examined whether the association between three-week averaged of temperature and methylation differs across quantiles of the methylation distributions in nine candidate genes. We measured gene-specific blood methylation repeatedly in 777 elderly men participating in the Normative Aging Study (1999–2010). We fit quantile regressions for longitudinal data to investigate whether the associations of temperature on methylation (expressed as %5mC) varied across the distribution of the methylation outcomes. We observed heterogeneity in the associations of temperature across percentiles of methylation in *F3*, *TLR-2*, *CRAT*, *iNOS*, and *ICAM-1* genes. For instance, an increase in three-week temperature exposure was associated with a longer left-tail of the *F3* methylation distribution. A 5°C increase in temperature was associated with a 0.15%5mC (95% confidence interval (CI): –0.27, –0.04) decrease on the 20th quantile of *F3* methylation, but was not significantly related to the 80th quantile of this distribution (Estimate:0.06%5mC, 95%CI: –0.22, 0.35). Individuals with low values of *F3*, *TLR-2*, *CRAT*, and *iNOS* methylation, as well as a high value of *ICAM-1* methylation, may be more susceptible to temperature effects on systemic inflammation.

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Institution review boards

This study was approved by the Harvard School of Public Health and the Veteran Administration Institution Review Boards (IRB). Participants provided written informed consent to participate in this study, which was approved by the Veteran Administration IRB.

Keywords

Quantile regression; temperature; DNA methylation; susceptibility

1. INTRODUCTION

Ambient temperature has been related to increased risk for cardiovascular mortality (Koken, et al. 2003). Mechanisms by which temperature causes cardiovascular mortality are not fully elucidated, but include the production of heat-shock proteins and inflammation (Moseley 1998). Moreover, inflammation has been related to cardiovascular disease (Bucova, et al. 2008; Lee, et al. 2011; Libby 2002). Recent research has pointed to epigenetics as a potential mechanism for the adverse effects of temperature (Bind, et al. 2014; Marsh and Pasqualone 2014). We recently conducted a mediation analysis in the Normative Aging Study cohort and found that the association between temperature and the intercellular adhesion molecule 1 (ICAM-1) protein was mediated via a change in *ICAM-1* methylation (Bind, et al. 2016). Epigenetics relates to changes in chromatin structure that influence gene expression, but do not modify the genetic code. The most frequently examined epigenetic mechanism is DNA methylation, which involves methylation of cytosine in CpG dinucleotides.

Three-week averaged temperature increase has been associated to increases in mean in DNA methylation measured on the *ICAM-1* and carnitine O-acetyltransferase (*CRAT*) genes and to decrease in mean methylation measured on the toll-like receptor 2 (*TRL-2*) gene (Bind, et al. 2014). This existing work used standard regression methods that estimate the change in the mean of an outcome for a given change in exposure. However, focus on the expected value of the response does not fully describe effects of exposure on the shape rather than the location, of the outcome distribution. DNA methylation is measured as the proportion of cells that carry methylated CpG sites at a given location. For several genes, particularly if related to cell signaling such as those on inflammatory pathways, the transition of subset of cells from a non-methylated to a methylated state – or vice versa – might result in the recruitment of a larger proportion of cells resulting in signal amplification. This process has been suggested to determine an apparent threshold effect in methylation dynamics as well as in the regulation of gene expression (Liu, et al. 2013). Because DNA methylation is a biological mechanism whereby cells control gene expression in this complex manner (Riggs and Xiong 2004), we hypothesized that mean regression analyses may not capture associations that occur primarily in one extreme of the outcome distribution, and therefore would miss exposure-outcome associations in susceptible groups.

This study investigates whether the association between temperature and DNA methylation varies across nine deciles of the methylation distribution (10th to 90th). Quantile regression is distribution free, thereby allowing one to avoid the assumption of normality of traditional mean regression methods. We focus on gene-specific methylation on nine candidate genes related to heart disease. For genes with low levels of methylation, we hypothesize that temperature decrease (e.g., in winter) or temperature increase (e.g., in summer) may be associated with methylation decrease that could lead to some increased production of

cardiovascular-related proteins. However, for gene with medium and high levels methylation, because of the complexity of the methylation machinery, it is less clear how temperature may affect genes methylation. We chose to study an elderly cohort that is susceptible to extreme temperatures (Kim and Joh 2006).

2. MATERIALS AND METHODS

2.1 Study population

Participants included in this analysis came from the Normative Aging Study (NAS), a longitudinal investigation established in Boston in 1963 by the U.S. Veterans Administration. (Bell, et al. 1966) We measured DNA methylation on a total of 777 individuals one to five times with intervals of three to five years ($n_{\text{observations}}=1,798$). We assessed DNA methylation on blood samples collected after smoking abstinence and an overnight fast over the 1999–2010 period. Participants with levels of C-reactive protein over 10 mg/L were excluded so that results are not confounded by infection state (Simon, et al. 2004).

2.2 Temperature

The time window of temperature exposure most relevant for changes in DNA methylation is unclear. A recent marine study suggested an association spread over four weeks (Marsh and Pasqualone 2014). Adult worms were cultured for four weeks at two temperatures (-1.5°C as ambient control and $+4^{\circ}\text{C}$ as warm treatment). About 3000 sites evidenced a change in methylation state and among them 85% involved a gain of a 5-methyl group on a CpG site (net increase in methylation). In the same cohort investigated in this study (i.e., Normative Aging Study), we observed associations between temperature averaged up to three weeks preceding the medical visit and the mean of gene-specific methylation distributions (Bind, et al. 2014). Therefore, we chose *a priori* to explore the same intermediate-term exposure window and focused on temperature averaged over the three-week period preceding each participant's methylation assessment. We examined only one exposure window to limit the number of tests.

We obtained measurements of ambient temperature from Boston Logan Airport weather station, which is located 8 km from the medical center. Since most of the participants live throughout the metropolitan area, we assumed that the monitored temperature can serve as a surrogate of their exposures.

2.3 DNA methylation

We collected participant's blood at every visit and isolated DNA to assess gene-specific DNA methylation using highly quantitative methods based on bisulfite polymerase chain reaction pyrosequencing (Baccarelli, et al. 2010a). Nine candidate genes that were expressed in leukocytes and plausibly related to cardiovascular disease were chosen for high precision pyrosequencing analysis *a priori* as part of a previous study. We previously examined the mean association between temperature and methylation on the same set of genes (Bind, et al. 2014).

We measured methylation levels in tissue factor (*F3*), intercellular adhesion molecule 1 (*ICAM-1*), toll-like receptor 2 (*TRL-2*), carnitine O-acetyltransferase (*CRAT*), 8-oxoguanosine DNA glycosylase-1 (*OGGI*), interferon gamma (*IFN-γ*), inducible nitric oxide synthase (*iNOS*), and glucocorticoid receptor (*GCR*) genes, at two to five CpG positions within each gene's promoter region (see more details in Table 1). The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines at position 5 (%5mC), and each gene-specific outcome was calculated as the mean values of the position-specific measurements. Interleukin 6 (*IL-6*) methylation was quantified outside the gene's promoter region.

2.4 Statistical methods

After ruling out any deviation from a linear relationship between temperature and mean methylation (using cubic splines), we investigated whether temperature levels averaged over the three-week period before assessment was linearly associated with nine deciles (10th to 90th) of the methylation distribution. In our regression models, the dependent variable was gene-specific DNA methylation. Because 70% of the participants had repeated methylation measures, we fit quantile regressions for longitudinal data and report the associations on the additive scale (Koenker 2004). Briefly, this approach fits fixed-effects and correlated-random-effects quantile regression models and relies on Bootstrap inference. Median regression chooses regression coefficients that minimize the sum of the absolute values of the residuals instead of the sum of squared residuals. The result is an estimate of covariate effects on the median, instead of the mean, of the outcome distribution. Quantile regression generalizes this approach by weighting the positive and negative residuals differently, which forces the regression line through other percentile of the distribution, with the exact percentile depending on the exact weighting of the residuals. We use the “rqpd” R package that allows for positive correlation between the repeated measures of outcomes (Koenker 2004).

We reported estimated quantile regression coefficients, which correspond to differences in methylation (expressed in %5mC) at that quantile associated with a 5°C increase in temperature. We adjusted for the following potential confounders: relative humidity, sine and cosine terms as a function of day of the season, and batch of methylation measurement. We also controlled for the following factors likely to influence methylation but not exposure: age, diabetes, body mass index, smoking status, statin use, as well as percentages of neutrophils, lymphocytes, monocytes, and basophils in differential blood count (Baccarelli, et al. 2010b).

We conducted sensitivity analyses that 1) examined different exposure time windows (24h-mean and 7-day moving average) and 2) adjusted for 3-week moving average of PM_{2.5} concentrations.

3. RESULTS

3.1 Descriptive statistics

At baseline, the median age of the participants was 72 years, 14% were diabetics, 4% were current smokers, and most were overweight or obese. Individuals' characteristics differed by their total number of visits: participants with more visits seemed healthier than participants with fewer visits (see Table 2).

Boston's climate is considered continental with direct influences from the ocean. While it is usually warm and humid in summer, it is mostly cold and dry in winter. The median temperature among the total number of observations was 13°C (5th percentile: -1°C; 95th percentile: 23°C).

The mean and deciles of the methylation distributions are presented in Table 3 and the empirical distributions in Figure 1. For *F3*, *TLR-2*, *CRAT*, *ICAM-1*, and *OGG* methylation levels were low (90th decile < 6.5%5mC) and their distributions were skewed. Methylation levels for *IFN-γ*, *IL-6*, *iNOS*, and *GCR* were medium or high and their distributions appeared to be less skewed.

3.2 Quantile regression results

Our results showed that the association between three-week average temperature and DNA methylation was not homogenous across quantiles of methylation measured on all nine genes (see Figure 1). While temperature was primarily related to *F3*, *TLR-2*, *CRAT*, *iNOS* methylation in the lower quantiles of these methylation distributions, we observed stronger associations between temperature and the highest percentiles of the *ICAM-1* methylation distribution. For instance, a 5°C increase in temperature was associated with a 0.15%5mC (95% confidence interval (CI): -0.27, -0.04) decrease on the 20th quantile of *F3* methylation, but was not significantly related to the 80th quantile (Estimate: 0.06%5mC, 95% CI: -0.22, 0.35). Similarly, a 5°C increase in temperature was related to a 0.61%5mC (95% CI: 0.25, 0.98) increase in the 80th quantile of *ICAM-1* methylation, but was not significantly related to the 20th quantile (Estimate: 0.06% 5mC, 95% CI: -0.20, 0.31). We did not find any heterogeneity nor significant associations between temperature and the percentiles of the *OGG1*, *IFN-γ*, *IL-6* and *GCR* methylation distributions.

When examining the 24h-mean exposure time window, we did not find any associations between temperature and deciles of methylation outcomes (except for the 20th decile of the *IFN-γ* methylation distribution). When investigating the 7-day moving average exposure time window, we found no heterogeneity for the temperature-methylation association, except for *F3* and *TLR-2*. We observed clearer evidence for temperature-methylation negative associations for *F3* among the lowest quantiles (i.e., 10th to 40th deciles) and for *TLR-2* among the higher quantiles (60th, 70th, and 80th deciles). When adjusting for 3-week moving average of PM_{2.5} concentrations, we found similar results as in the main quantile model.

4. DISCUSSION

Our results provide evidence of an association between temperature exposures and the shape of the distribution of DNA methylation. Our findings suggest that temperature is negatively associated with the lowest quantiles of the *F3* and *TLR-2* methylation distributions, positively associated with the highest quantiles of *ICAM-1* methylation, as well as positively related to the lowest quantiles of the *iNOS* and *CRAT* methylation distributions. Together, all of these associations indicate that temperature is associated with an elongation of the tails of the *F3*, *TLR-2*, and *ICAM-1* methylation distributions. These results also indicate that individuals with higher or lower methylation levels, depending on the gene, are more prone than the remaining participants to the effects of temperature on DNA methylation.

F3 (also called tissue factor) plays an important role in the coagulation cascade. (Holy and Tanner 2010) Elevated levels of *F3* have been observed in atherosclerotic plaques and have been suggested to be critical in the pathogenesis of atherothrombosis (Jude, et al. 2005). Since reduced methylation is generally associated with increased expression, our results suggest *F3* levels may be increased by warm temperature. Unfortunately, protein levels were not available to confirm this. It could be interesting to confirm that in response to temperature, there is some thrombus formation activated by a decrease in *F3*, which could lead to the pathogenesis of atherosclerosis.

Temperature increases were associated with *TLR-2* hypomethylation at the 10th to 70th percentiles. *TLR-2* is a gene that codes a protein involved in innate immunity. While *TLR-2* may be related to IL-6 (Shoenfelt, et al. 2009), elevated IL-6 concentrations have been associated with increased C-reactive protein levels (Van Gaal, et al. 2006). Previous studies have reported increases in C-reactive protein related to higher temperatures (Hampel, et al. 2010; Schauble, et al. 2012; Wilker, et al. 2012). Therefore, after exposure to warm temperatures, *TLR-2* hypomethylation may activate *TLR-2* gene expression and induce biological responses activating the expression of C-reactive protein. Again, it would be interesting to confirm this statement with a future study.

In this study, high temperatures were associated with increased levels of *ICAM-1* methylation only in the highest quantiles of the *ICAM-1* methylation distribution. Since the temperature-methylation association was modeled linearly, this also suggests that low temperatures were associated with a decrease in *ICAM-1* methylation only in the upper tail of the *ICAM-1* methylation distribution. In our data, a decrease in *ICAM-1* methylation was also directly associated with increased *ICAM-1* protein. *ICAM-1* is a protein expressed on cells of the immune system and endothelial cells. High concentrations in *ICAM-1* have been shown to increase the risk of heart disease (Luc, et al. 2003). Our results suggest that low temperatures may decrease *ICAM-1* methylation, which may cause *ICAM-1* gene de-silencing and an increase in *ICAM-1* protein expression.

Similarly, high temperature was positively associated with *iNOS* methylation and low temperature was associated with a decrease in *iNOS* methylation at the 20th to 40th percentiles. *iNOS* generates nitric oxide (NO) which plays an important role in inflammation and regulation of blood pressure regulation (Lechner, et al. 2005). In the same NAS cohort,

air-pollution was also related to *iNOS* hypomethylation (Madrignano, et al. 2011). Thus, cold temperatures may lead *iNOS* hypomethylation, which may also induce an increase in *iNOS* protein expression.

Moreover, high temperature was related to an increase in *CRAT* methylation. *CRAT* participates in the metabolic pathway in mitochondria, peroxisomes, and endoplasmic reticulum. It therefore constitutes a key enzyme in the cellular cycle. A previous study suggested that *CRAT* concentrations are associated with increased risk of cardiovascular outcomes (Brunner, et al. 1997). In the same NAS cohort, *CRAT* hypermethylation was related to aging (Madrignano, et al. 2012). This study suggests that high temperatures may induce *CRAT* hypermethylation, which could be another cellular metabolic mechanism that explains the relationship between high temperatures and cardiovascular-related death.

Our results are fairly consistent with previous analyses based on mean regression and distributed-lag models for the exposure in the same cohort (Bind, et al. 2014). In both studies, low temperatures were associated with *ICAM-1* hypomethylation and high temperatures were associated with *TLR-2* and *CRAT* hypomethylation. Temperature was not significantly related to *OGG1*, *IFN- γ* , *IL-6*, *TLR-2*, and *GCR* methylation in both studies. This quantile analysis reveals that the previous mean associations were driven by one extreme of the methylation distributions, and no change occurred at the opposite end of the distribution. In the previous study using standard mean regression analysis, temperature was not associated with *F3* and *iNOS* mean methylation, while this quantile analysis reveals significant temperature associations with the extremes of the *F3* and *iNOS* methylation distributions. This result demonstrates the added value of the quantile regression approach.

This estimation method examining quantiles allows us to describe effects that shift the overall shape of the outcome distribution. For example, while we observed that temperature shifts the low quantiles of the *F3* methylation distribution towards lower levels, we found no significant association on the upper quantiles. This finding suggests that individuals with low *F3* methylation may be more susceptible to temperature. The left-tail of the *F3* methylation distribution under high temperature exposure becomes longer than would be expected under low temperature conditions. In contrast, for *ICAM-1* methylation, the impact of low temperature is primarily on the upper quantiles, and so with colder temperature, the right tail of the distribution is reduced. Hence, in this case, the effect of cold temperature is to increase the number of people at risk of higher expression, rather than to increase the extent of higher expression in those already at risk.

Limitations and strengths

Participants in this study belong to the older category of the US male and mostly white population. Survival bias might be present in the estimates we obtained. However, in the same cohort, we investigated the air pollution association with DNA methylation and found similar estimates after adjusting our estimates with censoring inverse probability weights (Bind, et al. 2014). Generalizability to women, younger people, or people with other ethnicity is unclear. Measurement error due to the single site monitoring of temperature us also likely to have biased the results downwards. In addition, we reported temperature-methylation associations across deciles of the outcomes. Future work could examine

whether the quantile-specific estimates change when considering causal models for observational studies.

To our knowledge, this is the first study to examine distributional changes on epigenetic outcomes associated with temperature changes. Quantile regression does not specify any distribution for the residuals, and hence is distribution free. Quantile regression also allows us to obtain regression coefficient estimates on the additive scale and therefore directly interpretable in the units of the outcome. In contrast, standard methods often require assumptions about the distribution of the methylation data or the residuals of a linear mean model.

5. CONCLUSIONS

This study allowed us to detect some distributional changes in methylation due to temperature and quantify some thresholds of DNA methylation, at which elderly men may be more susceptible to temperature changes. In the case of *F3*, *TLR-2*, *CRAT*, *iNOS* methylation, the association between these outcomes and temperature was concentrated on participants in the lower quantiles of the methylation distribution, that is, the ones who had low levels of methylation. Quantile regression can yield evidence about mechanisms linking exposure and outcome. For example, this study suggested that temperature is sometimes associated with only one extreme of methylation distributions; which may also indicate individual epigenetic susceptibility. In presence of heterogeneous effects along the outcome distribution, we recommend to report the quantile estimates to yield a fuller picture of the association between the exposure and the distribution of the outcome of interest.

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Highlights

- This study allowed us to detect some distributional changes in methylation associated with temperature.
- For *F3*, *TLR-2*, *CRAT*, *iNOS* methylation, the associations between these outcomes and temperature were concentrated on participants in the lower quantiles of the methylation distribution, that is, the ones who had low levels of methylation.
- Our study suggested that temperature is associated with only one extreme of some methylation distributions; which may also indicate individual epigenetic susceptibility.
- In presence of heterogeneous effects along the outcome distribution, we recommend to report the quantile estimates to yield a fuller picture of the association between the exposure and the distribution of the outcome of interest.

Figure 1.
Empirical distribution of the nine methylation outcomes

Figure 2.
Difference in gene-specific methylation (%5mC) for an 5°C increase in 3-week moving average temperature across quantiles of methylation

Figure 3.

a: Difference in gene-specific methylation (%5mC) for an 5°C increase in 24h-mean temperature across quantiles of methylation. b: Difference in gene-specific methylation (%5mC) for an 5°C increase in 7-day moving average temperature across quantiles of methylation

Figure 4.
Difference in gene-specific methylation (%5mC) for an 5°C increase in temperature across quantiles of methylation adjusted for PM_{2.5}

Table 1

Location of the gene promoter, region amplified, and CpG sites

Gene	Chromo some	Promoter Start end	Amplicon Start end	Location	Promoter	CpGs (position)
<i>F3</i>	1	94779671 94780502	94779878 94780068	CpG island	Yes	94779947 (pos1) 94779950 (pos2) 94779956 (pos3) 94779958 (pos4) 94779974 (pos5)
<i>TLR-2</i>	4	154824391 154824991	154824566 154824754	CpG island	Yes	154824709 (pos1) 154824713 (pos2) 154824715 (pos3) 154824723 (pos4) 154824727 (pos5)
<i>CRAT</i>	9	130912702 130913404	130912776 130912862	CpG island	Yes	130912824 (pos1) 130912806 (pos2)
<i>ICAM-1</i>	19	10242017 10242937	10242034 10242283	CpG island	Yes	10242236 (pos1) 10242225 (pos2) 10242218 (pos3)
<i>OGGI</i>	3	9766128 9766775	9766288 9766514	CpG island	Yes	9766356 (pos1) 9766366 (pos2) 9766373 (pos3) 9766380 (pos4)
<i>IFN-γ</i>	12	66839561 66840293	66840120 66840260	non-CpG island		66840192 (pos1) 66840186 (pos2)
<i>IL6</i>	7	22732791 22733685	22733758 22733893	non-CpG island	No	22733847 (pos1) 22733841 (pos2)
<i>iNOS</i>	17	23149861 23150461	23149873 23149990	non-CpG island	Yes	23149929 (pos1) 23149936 (pos2)
<i>GCR</i>	5	142760496 142761097	142760531 142760806	non-CpG island	Yes	142760565

Table 2
Demographical characteristics of the Normative Aging Study participants across visits

Visits Age	(years) [5 th , 50 th , 95 th percentiles]	Body mass index [5 th , 50 th , 95 th percentiles]	Statin User	Diabetic	Smoking Status [Never, Former, Current]
Baseline (n=777)	[62, 72, 84]	[23, 28, 35]	36%	14%	[29%, 67%, 4%]
N _{missing}	0	0	0	0	0
Among participants having one visit (n ₁ =221)					
Visit 1	[64, 76, 88]	[22, 28, 36]	40%	18%	[26%, 70%, 4%]
Among participants having two visits (n ₂ =217)					
Visit 1	[60, 73, 83]	[23, 28, 36]	35%	16%	[26%, 69%, 5%]
Visit 2	[66, 77, 86]	[22, 27, 37]	54%	19%	[26%, 70%, 4%]
Among participants having three visits (n ₃ =216)					
Visit 1	[62, 71, 82]	[23, 28, 35]	36%	9%	[29%, 68%, 3%]
Visit 2	[66, 74, 86]	[22, 28, 34]	52%	13%	[28%, 69%, 3%]
Visit 3	[69, 78, 89]	[22, 27, 35]	62%	17%	[27%, 71%, 2%]
Among participants having four visits (n ₄ =120)					
Visit 1	[60, 69, 77]	[23, 28, 34]	29%	10%	[38%, 58%, 4%]
Visit 2	[63, 72, 81]	[23, 27, 34]	42%	11%	[38%, 58%, 4%]
Visit 3	[66, 75, 84]	[22, 27, 33]	59%	16%	[38%, 59%, 3%]
Visit 4	[70, 78, 87]	[22, 27, 34]	65%	18%	[38%, 60%, 2%]

Note: Three individuals had 5 visits and their characteristics were fairly better than the other participants

Table 3
Means and deciles of the gene-specific methylation distributions (expressed in %5mC)

Gene	N _{total}	Mean	10 th	20 th	30 th	40 th	Median	60 th	70 th	80 th	90 th
F3	1533	2.3	1.1	1.3	1.6	1.8	2.1	2.4	2.7	3.1	3.7
TLR-2	1424	2.9	1.4	1.8	2.1	2.3	2.6	3.0	3.4	3.9	4.7
CRAT	1669	3.6	1.9	2.2	2.7	3.0	3.3	3.5	3.8	4.4	5.9
ICAM-1	1424	4.3	2.5	3.0	3.3	3.7	4.0	4.4	4.8	5.3	6.3
OGG	1159	2.1	0.7	1.0	1.3	1.5	1.7	2.1	2.5	3.0	3.9
IFN-γ	1736	84.8	78.0	80.9	83.1	84.4	85.8	86.9	87.8	88.9	90.1
IL-6	1749	43.3	30.0	35.0	38.6	41.5	43.6	45.6	48.1	51.1	56.2
iNOS	1273	68.1	58.7	62.7	65.19	67.0	69.0	70.6	72.3	74.2	76.6
GCR	1549	46.9	40.2	42.9	44.6	46.0	47.1	48.3	49.6	51.0	53.2