Differential methylation between ethnic sub-groups reflects the effect of genetic ancestry and environmental exposures

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Sources of Funding:

This research was supported in part by the Sandler Family Foundation, the American Asthma Foundation, National Institutes of Health (P60 MD006902, R01 HL117004, R21ES24844, U54MD009523, R01 ES015794, R01 HL088133, M01 RR000083, R01 HL078885, R01 HL104608, U19 AI077439, M01 RR00188, U01 HG009080, and R01 HL135156), ARRA grant RC2 HL101651, and TRDRP 24RT-0025; EGB was supported in part through grants from the Flight Attendant Medical Research Institute (FAMRI), and NIH (K23 HL004464); NZ was supported in part by an NIH career development award from the NHLBI (K25HL121295). JMG was supported in part by NIH Training Grant T32 (T32GM007546) and career development awards from the NHLBI (K23HL111636) and NCATS (KL2TR000143) as well as the Hewett Fellowship; N.T. was supported in part by an institutional training grant from the NIGMS (T32-GM007546) and career development awards from the NHLBI (K12-HL119997 and K23-HL125551), Parker B. Francis Fellowship Program, and the American Thoracic Society; CRG was supported in part by NIH Training Grant T32 (GM007175) and the UCSF Chancellor's Research Fellowship and Dissertation Year Fellowship; RK was supported with a career development award from the NHLBI (K23HL093023); HJF was supported in part by the GCRC (RR00188); PCA was supported in part by the Ernest S. Bazley Grant; MAS was supported in part by 1R01HL128439-01. This publication was supported by various institutes within the National Institutes of Health. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

One Sentence Summary

In order to better understand the role of ethnic self-identification and genetically determined ancestry in biomedical outcomes, we explore their relative contributions to variation in methylation, a fundamental biological process.

Abstract

In clinical practice and biomedical research populations are often divided categorically into distinct racial/ethnic groups. In reality, these categories, which are based on social rather than biological constructs, comprise diverse groups with highly heterogeneous histories, cultures, traditions, religions, social and environmental exposures and ancestral backgrounds. Their use is thus widely debated and genetic ancestry has been suggested as a complement or alternative to this categorization. However, few studies have examined the relative contributions of racial/ethnic identity, genetic ancestry, and environmental exposures on well-established and fundamental biological processes. We examined the associations between ethnicity, ancestry, and environmental exposures and DNA methylation. We typed over 450,000 CpG sites in primary whole blood of 573 individuals of diverse Hispanic descent who also had high-density genotype data. We found that both self-identified ethnicity and genetically determined ancestry were significantly associated with methylation levels at a large number of CpG sites (916 and 194, respectively). Among loci differentially methylated between ethnic groups, a median of 75.7% (IQR 45.8% to 92%) of the variance in methylation associated with ethnicity could be accounted for by shared genomic ancestry accounts. We also found significant enrichment ($p = 4.2 \times 10^{-64}$) of ethnicity-associated sites amongst loci previously associated with environmental and social exposures, particularly maternal smoking during pregnancy. Our study suggests that although differential methylation between ethnic groups can be partially explained by the shared genetic ancestry, a significant effect of ethnicity is likely due to environmental, social, or cultural factors, which differ between ethnic groups.

Introduction

Race, ethnicity, and genetic ancestry have had a complex and often controversial history within biomedical research and clinical practice[1-3]. For example, race- and ethnicity-specific clinical reference standards are based on population-based sampling on a given physical trait

30 such as pulmonary function[4,5]. However, because race and ethnicity are social constructs
31 and poor markers for genetic diversity, they fail to capture the heterogeneity present within
32 racial/ethnic groups and in admixed populations[6]. To account for these heterogeneities and
33 to avoid social and political controversies, the genetics community has grouped individuals by
34 genetic ancestry instead of race and ethnicity[3]. Indeed, recent work from our group and
35 others have demonstrated that genetic ancestry improves diagnostic precision compared to
36 racial/ethnic categorizations for specific medical conditions and clinical decisions[7-9].

37 However, racial and ethnic categories also reflect the shared experiences and exposures to
38 known risk factors for disease, such as air pollution and tobacco smoke, poverty, and
39 inadequate access to medical services, which have all contributed to worse disease outcomes in
40 certain populations[10,11]. Thus, it is unclear whether defining groups through genetic
41 ancestry can capture these shared exposures. In this work we seek to explore the relative
42 contributions of genetically defined ancestry and social, cultural and environmental factors to
43 understanding differential methylation between ethnic groups.

44 Epigenetic modification of the genome through methylation plays a key role in the regulation
45 of diverse cellular processes[12]. Changes in DNA methylation patterns have been associated
46 with complex diseases, including various cancers[13], cardiovascular disease[14,15],
47 obesity[16], diabetes[17], autoimmune and inflammatory diseases[18], and neurodegenerative
48 diseases[19]. Epigenetic changes are thought to reflect influences of both genetic[20] and
49 environmental factors[21], and have been shown to vary between racial groups[22]. The
50 discovery of methylation quantitative trait loci (meQTL's) across populations by Bell et al.
51 established the influence of genetic factors on methylation levels in a variety of tissue
52 types[20], with meQTL's explaining between 22% and 63% of the variance in methylation
53 levels. Multiple environmental factors have also been shown to affect methylation levels,
54 including endocrine disruptors, tobacco smoke[23,24], polycyclic aromatic hydrocarbons,
55 infectious pathogens, particulate matter, diesel exhaust particles[25], allergens, heavy metals,
56 and other indoor and outdoor pollutants[26]. Psychosocial factors, including measures of
57 traumatic experiences[27-29], socioeconomic status[30,31], and general perceived stress[32],
58 also affect methylation levels. Since both genetic and environmental exposures affect

methylation, this represents an ideal phenotype to explore the relative contributions of these two factors on differential methylation between ethnic groups.

In this work, we leveraged genome-wide methylation data in 573 Latino children of diverse Latino sub-ethnicities enrolled in the Genes-Environment and Admixture in Latino Americans (GALA II) study[33] whose genetic ancestry had been determined from dense genotyping arrays. This allowed us to explore the extent to which the differences in methylation between Latino sub-groups could be explained by their shared genetic ancestry. We found that many of the methylation differences associated with ethnicity could be explained by shared genetic ancestry. However, even after adjusting for ancestry, significant differences in methylation remained between the groups remained at multiple loci, reflecting social and environmental influences upon methylation.

Our findings have important implications for both the use of ancestry to capture biological changes and of race/ethnicity to account for social and environmental exposures. Epigenome-wide association studies in diverse populations may be susceptible to confounding due to environmental exposures in addition to confounding due to population stratification[34]. The findings also have implications for the common practice of considering individuals of Latino descent, regardless of origin as a single ethnic group.

Results

The study included 573 participants, the majority of whom self-identified as being either of Puerto Rican (n = 220) or Mexican origin (n = 276). Table 1 displays baseline characteristics of the GALA II study participants with methylation data included in this study, stratified by ethnic subgroups (Puerto Rican, Mexican, Other Latino, and Mixed Latinos who had grandparents of more than one national origin). Figure S1 shows the distribution of African, European, and Native American ancestry among the 524 participants with genomic ancestry estimates.

Global patterns of methylation

Differences in ethnicity and ancestry resulted in discernible patterns in the global methylation profile as demonstrated in a multidimensional scaling analysis [Figure S2A]. As expected[30,35], the first few principal coordinates are strongly correlated to imputed cell

88 composition [Figure S2B-C]. There are also significant associations of self-identified sub-
89 ethnicity with PC2 (p-ANOVA = 0.003), PC3 (p-ANOVA = 0.004), PC6 (p-ANOVA = 0.0001), PC7 (p-
90 ANOVA = 0.0003) [Figure S3A], and PC8 (p-ANOVA = 0.0003), after adjusting for age, sex, disease
91 status, cell components, and technical factors (plate and position). Genetic ancestry was
92 associated with PC3 (p-ANOVA = 0.002), PC7 (p-ANOVA = 0.0004) [Figure S3B] and PC8 (p-
93 ANOVA = 0.001) in a two degree of freedom ANOVA test, adjusting for age, sex, disease status,
94 cell components, technical factors, and ethnicity. Table S1 summarizes the results of the
95 simple correlation analysis of methylation with ethnicity and ancestry, as well as the adjusted
96 nested ANOVA models described above and the mediation results described below.

97 A mediation analysis[36] revealed that the associations between ethnicity and PCs 3, 7, and 8
98 were significantly mediated by Native American ancestry (mediation p = 0.01, <0.001, and
99 <0.001, respectively) and inclusion of Native American ancestry in the regression model of PCs
100 3, 7, and 8 caused the ethnicity associations to be non-significant. However, the associations of
101 ethnicity with PCs 2 and 6 were not explained by Native American, African or European
102 ancestry (mediation p > 0.05), suggesting that the ethnic differences in these principal
103 components are associated with global methylation patterns not captured by the shared
104 genetic ancestry of each ethnic group. When genetic ancestry was regressed on the
105 methylation data with the principal coordinates recalculated using the residuals of the
106 regression between methylation and ancestry, there was an association between ethnicity and
107 PC6 (p-ANOVA = 0.003). However, there was no association with any of the other principal
108 coordinates. These observations suggest that while shared genetic ancestry can explain some
109 of the association between ethnicity and global methylation patterns, other non-genetic
110 factors, such as environmental and social exposure differences associated with ethnicity
111 influence methylation and are not captured by measures of genetic ancestry.

112 Epigenome-wide association of self-identified ethnicity

113 An epigenome-wide association study of self-identified ethnicity (see methods for details of
114 ascertainment of ethnicity) and methylation identified a significant difference in methylation
115 M-values between ethnic groups at 916 CpG sites at a Bonferroni-corrected significance level of
116 less than 1.6×10^{-7} [Figure 1A and Table S2]. The most significant association with ethnicity
117 occurred at cg12321355 in the ABO blood group gene (*ABO*) on chromosome 3 (p-ANOVA $6.7\times10^{-}$

118 [22]) [Figure 1B]. A two degree of freedom ANOVA test for genomic ancestry was also
119 significantly associated with methylation level at this site ($p = 2.3\times10^{-5}$) [Figure 1C], and when
120 the analysis was stratified by ethnic sub-group, showed an association in both Puerto Ricans
121 and Mexicans ($p = 0.001$ for Puerto Ricans, $p = 0.003$ for Mexicans). Although adjusting for
122 genomic ancestry attenuated the effect of ethnicity, a significant association between ethnicity
123 and methylation remained ($p = 0.04$). Recruitment site, an environmental exposure proxy, was
124 not significantly associated with methylation at this locus ($p = 0.5$), suggesting that
125 environmental differences associated with ethnicity beyond geography and ancestry are
126 driving the association.

127 In order to determine the relative contribution of shared genetic ancestry and other factors
128 associated with ethnicity, we repeated the analysis adjusting for ancestry. A significant
129 association remained in 314 of the 834 (37.8%, $p = 1.7 \times 10^{-183}$ for enrichment) CpG sites
130 associated with ethnicity [Figure S4A and Table S2] (82 sites were excluded because they
131 demonstrated unstable coefficient estimates and inflated standard errors due to strong
132 correlations between ethnicity and ancestry, especially Native American ancestry [see Figure
133 S1]). Genomic ancestry explained a median of 4.2% (IQR 1.8% to 8.3%) of the variance in
134 methylation at these loci and accounts for a median of 75.7% (IQR 45.8% to 92%) of the total
135 variance in methylation explained jointly by ethnicity and ancestry [Figure S4B]. Sensitivity
136 tests for departures from linearity, fine scale population substructure and the exclusion of the
137 16 participants who self-identified as "Mixed Latino" sub-ethnicity, did not meaningfully affect
138 our results [See Supplementary Text and Tables S2 – S6]. To rule out any residual confounding
139 due to recruitment sites, we conducted an additional analysis on the effect of recruitment site
140 on methylation both for the overall study and for the Mexican participants (the largest study
141 population in this analysis). We observed no significant independent effect of recruitment site
142 suggesting that confounding due to recruitment region was limited, at least within the United
143 States.

144 To explore the effect of departures from a linear association between ancestry and
145 methylation, we incorporated both higher order polynomials and cubic splines of ancestry into
146 our models. We observed a significant departure from linearity ($p < 0.05$) in only 26 (for
147 splines) and 25 (for polynomials) of the 314 CpG's where an association between ethnicity and

148 methylation remained after adjusting for ancestry; however, the association between ethnicity
149 and methylation remained even after adjusting for non-linearity at all sites [Tables S3 and S4].

150 Environmental differences between geographic locations or recruitment sites are a potential
151 non-genetic explanation for ethnic differences in methylation. We investigated the
152 independent effect of recruitment site on methylation by analyzing the associations between
153 recruitment site and individual methylation loci after adjusting for ethnicity. We did not find
154 any loci significantly associated with recruitment site at a significance threshold of 1.6×10^{-7}.
155 We then performed an analysis to assess the effect of recruitment sites on methylation
156 stratified by ethnicity. We did not find any loci significantly associated with recruitment site
157 and methylation among Mexican participants. We were underpowered to perform a similar
158 analysis for Puerto Ricans because there were only 27 Puerto Rican participants recruited
159 outside of Puerto Rico. To ensure that the absence of association in Mexicans was not due to
160 the loss of power from the smaller sample size, we repeated our analysis of the association
161 between ethnicity and ancestry randomly down-sampling to 276 participants to match the
162 sample size in the analysis of geography in Mexicans. While down-sampling the study to this
163 degree resulted in a loss of power, 128 methylation sites were still associated with ancestry. We
164 conclude that recruitment site was unlikely to be a significant confounder of our associations
165 between ethnicity and methylation and was not a significant independent predictor of
166 methylation.

167 While most population substructure in Latinos would be expected to arise from differences in
168 continental ancestry[37,38], there is evidence of finer scale (sub-continental) ancestry in
169 Latino populations[39]. We tested for the effect of fine scale substructure by calculating
170 principal components for all participants with genotyping data using Eigensoft[40]. We found
171 significant associations between principal components 3-10 (PC's 1 and 2 were almost perfectly
172 collinear with ancestry, with an adjusted $R^2 > 0.998$ for all three ancestry proportions, and were
173 therefore excluded) and ethnicity. We therefore added these 8 PC's to models of ethnicity and
174 methylation, and found an association between these genetic PC's and methylation in 63/314
175 CpG's that had remained associated with ethnicity after adjusting for ancestry. Adjusting for
176 higher order substructure in these CpG's explained the association between ethnicity and
177 methylation in 51 additional loci. This left 263 loci associated with ethnicity after adjustment

for ancestry where there was either no association between PC's 3-10 and methylation or the inclusion of these PC's did not affect the association between ethnicity and methylation.[Table S5]

As only 16 participants self-identified as "Mixed Latino", we performed a sensitivity analysis to test the effect of excluding these participants from the analysis and only examining Puerto Ricans, Mexicans, and "Other Latinos". We found that excluding self-identified "Mixed Latino" participants from the analysis did not significantly alter the results in most cases [Table S6]. Of the 916 CpG's associated with ethnicity at a genome-wide scale ($p < 1.6 \times 10\text{-}7$) in models including individuals self-identified as "Mixed Ethnicity", 894 (97.5%) were still significant at a genome-wide scale when "Mixed Latinos" were excluded. All but two of the CpG's that did not meet genome-wide significance were significant when correcting for 916 tests ($p < 5\times10\text{-}5$). In addition, an additional 290 CpG loci that did not meet genome-wide significance in the original analysis were significant at a genome-wide scale when self-identified "Mixed Latinos" were excluded. While these loci did not meet genome-wide significance in the original analysis that included Mixed Latinos, they all had p-values lower than 2×10^{-6}. Thus we conclude that a sensitivity test excluding individuals of mixed Latino ethnicity did not significantly alter the conclusions.

We conclude that shared genetic ancestry explains much but not all of the association between ethnicity and methylation. Other, non-genetic factors associated with ethnicity likely explain the ethnicity-associated methylation changes that cannot be accounted for by genomic ancestry alone.

Ethnic differences in environmentally-associated methylation sites

Methylation at CpG loci that had previously been reported to be associated with environmental exposures whose exposure prevalence differs between ethnic groups were tested for association with ethnicity in this study. A recent meta-analysis of maternal smoking during pregnancy, an exposure that varies significantly by ethnicity[33], identified associations with methylation at over 6,000 CpG loci[24]. We found 1341 of 4404 that passed QC in our own study (30.4%) were nominally associated with ethnicity ($p < .05$), which represented a highly significant ($p < 2\times10^{-16}$) enrichment. Using a Bonferroni correction for the 4404 loci tested, 126 maternal-smoking related loci were associated with ethnicity ($p < 1.1\times10^{-}$

^{5}), and 27 loci were among the 916 CpG's reported above as associated with ethnicity [Table S7]. We also examined methylation loci from an earlier study of maternal smoking in Norwegian newborns[23] as well as studies of diesel exhaust particles[25] and exposure to violence[27]. These results are supportive of our hypothesis that environmental exposures may be responsible for the observed differences in methylation between ethnic groups and are presented in Table S8.

In an earlier study of maternal smoking in Norwegian newborns[23] that identified 26 loci associated with maternal smoking during pregnancy, 19 passed quality control (QC) in our own analysis, and the association between methylation and ethnicity was found to be nominally significant ($p < 0.05$)at 6 (31.6%) CpG loci. Adjusting for 19 tests ($p < .0026$), cg23067299 in the aryl hydrocarbon receptor repressor (*AHRR*) gene on chromosome 5 remained statistically significant [Table S8]. These results suggest that ethnic differences in methylation at loci known to be responsive to tobacco smoke exposure *in utero* may be explained in part by ethnic-specific differences in the prevalence of maternal smoking during pregnancy.

We also found that CpG loci previously reported to be associated with diesel-exhaust particle (DEP) exposure[25] were significantly enriched among the set of loci whose methylation levels varied between ethnic groups. Specifically, of the 101 CpG sites that were significantly associated with exposure to DEP and passed QC in our dataset, 31 were nominally associated with ethnicity ($p < 0.05$), and 5 were associated with ethnicity after adjusting for 101 comparisons ($p < 0.005$) . Finally, we found that methylation levels at cg11218385 in the pituitary adenylate cyclase-activating polypeptide type I receptor gene (*ADCYAP1R1*), which had been associated with exposure to violence in Puerto Ricans[27] and with heavy trauma exposure in adults[28], was significantly associated with ethnicity ($p = 0.02$).

We also found 194 loci with a significant association between global genetic ancestry and methylation levels (after adjusting for ethnicity) at a Bonferroni corrected association p-value of less than 1.6×10^{-7} [Figure S5 and Table S9], including 48 that were associated with ethnicity in our earlier analysis. Of these significant associations, 55 were driven primarily by differences in African ancestry, 94 by differences in Native American ancestry, and 45 by differences in European ancestry. The most significant association between methylation and ancestry occurred at cg04922029 in the Duffy antigen receptor chemokine gene (DARC) on

238 chromosome 1 (ANOVA p-value 3.1×10^{-24}) [Figure S5B]. This finding was driven by a strong
239 association between methylation level and global African ancestry; each 25 percentage point
240 increase in African ancestry was associated with an increase in M-value of 0.98, which
241 corresponds to an almost doubling in the ratio of methylated to unmethylated DNA at the site
242 (95% CI 0.72 to 1.06 per 25% increase in African ancestry, $p = 1.1\times10^{-21}$). There was no significant
243 heterogeneity in the association between genetic ancestry and methylation between Puerto
244 Ricans and Mexicans (p-het = 0.5). Mexicans have a mean unadjusted methylation M-value 0.48
245 units lower than Puerto Ricans (95% CI 0.35 to 0.62 units, $p = 1.1\times10^{-11}$). However, adjusting for
246 African ancestry accounts for the differences in methylation level between the two sub-groups
247 (p-adjusted = 0.4), demonstrating that ethnic differences in methylation at this site are due to
248 differences in African ancestry.

249 The distribution of methylation M-values at cg04922029 is tri-modal, raising the possibility
250 that a SNP whose allele frequency differs between African and non-African populations may be
251 driving the association. We therefore looked at the association between methylation at
252 cg0422029 and ancestry at that locus. We found almost almost perfect correlation between
253 methylation and African ancestry at the locus ($p = 6\times10^{-162}$) [Figure 2A]. Each African haplotype
254 at the CpG site was associated with an increase in methylation M-value of 2.7, corresponding to
255 a 6.5-fold increase in the ratio of methylated to unmethylated DNA per African haplotype at
256 that locus. We then looked for SNPs within 10,000 base pairs of the CpG site that explained the
257 admixture mapping association. We found that methylation at cg04922029 was significantly
258 correlated with SNP rs2814778 [Figure 2B], the Duffy null mutation, 212 base pairs away; each
259 copy of the C allele was associated with an increase in M-value of 1.5, or a 2.9-fold increase in
260 the ratio of methylated to unmethylated DNA ($p = 3.8\times10^{-90}$) [Figure 2C].

261 When we examined the effect of local ancestry at the other 194 CpG's we find that a substantial
262 proportion of the effect of global ancestry on local methylation levels is due to local ancestry
263 acting in -cis. Among the 194 CpG sites associated with global ancestry, local ancestry at the
264 CpG site explained a median of 10.4% (IQR 3.0% to 19.4%) of the variance in methylation,
265 accounting for a median of 52.8% (IQR 20.3% to 84.9%) of the total variance explained jointly by
266 local and global ancestry [Figure S6].

Discussion

In a diverse population of Latinos, we have shown that a substantial number of loci are differentially methylated between ethnic sub-groups. While genomic ancestry can explain a portion of the association between ethnicity and methylation, factors other than shared ancestry that correlate with ethnicity, such as social, economic, cultural and environmental exposures account for the association between ethnicity and methylation at 34% (314/916) of loci.

We conclude that systematic environmental differences between ethnic subgroups likely play an important role in shaping the methylome for both individuals and populations. Loci previously associated with diverse environmental exposures such as *in utero* exposure to tobacco smoke[23,24], as well as diesel exhaust particles[25] and psychosocial stress[27] were enriched in our set of loci where methylation was associated with ethnicity. Twenty-seven of the loci associated with maternal smoking during pregnancy in a large consortium meta-analysis[24] were differentially methylated between Latino sub-groups at a genome-wide significance threshold of 1.6×10^{-7}. Thus, inclusion of relevant social and environmental exposures in studies of methylation may help elucidate racial/ethnic disparities in disease prevalence, health outcomes and therapeutic response. However, in many cases, a detailed environmental exposure history is unknown, unmeasurable or poorly quantifiable, and race/ethnicity may be a useful, albeit imperfect proxy.

Our comprehensive analysis of high-density methyl- and genotyping from genomic DNA allowed us to investigate the genetic control of methylation in great detail and without the potential destabilizing effects of EBV transformation and culture in cell lines[41]. The strongest patterns of methylation are associated with cell composition in whole blood[30]. However, the specific type of Latino ethnic-subgroups (Puerto Rican, Mexican, other, or mixed) is also associated with principal coordinates of genome-wide methylation.

Our approach has some potential limitations. It is possible that fine-scale population structure (sub-continental ancestry) within European, African, and Native American populations may contribute to ethnic differences in methylation, as we had previously reported in the case of lung function[39]. However, despite the presence of additional substructure among the GALA II participants, PC's 3-10 explained the association between ethnicity and ancestry at only 51

loci. PCs from chip-based genotypes will not capture all forms of genetic variation. Clusters of ethnicity specific rare variants of large effect or strong ethnicity-specific selective sweeps in the last 8-12 generations[37] could also give rise to methylation differences, but these are inconsistent with existing rare variant and selection analyses[42,43]. Our models of genetic ancestry assumed a linear effect of ancestry on methylation, whereas a nonlinear association or other model misspecification could have led to incomplete adjustment for genetic ancestry, and thus, led to a residual association between ethnicity and methylation. However, when we added second and third order polynomials or cubic splines to our models, we found evidence for a nonlinear association between ancestry and methylation at only 25 and 26 loci, respectively, and it did not affect the association between ethnicity and methylation. Although it is impossible to account for all types of non-linearity and non-additivity (such as gene by gene or gene by environment interaction), our analysis suggests that non-linear effects are unlikely to be significant. Since our study was geographically diverse, recruiting participants at five recruitment sites in the United States and Puerto Rico, it is possible that systematic differences associated with site of recruitment might have influenced observed methylation differences between ethnic groups. However, when we included recruitment site as a covariate, we found no significant effect on methylation independent of ethnicity.

The presence of a strong association between genetic ancestry and methylation raises the possibility that epigenetic studies can be confounded by population stratification, similar to genetic association studies, and that adjustment for either genetic ancestry or selected principal components is warranted. This possibility was first demonstrated in a previous analysis of the association between self-described race and methylation[22]. However, the study only evaluated two distinct racial groups (African Americans and Whites), while the present study demonstrates the possibility of population stratification in an admixed and heterogeneous population with participants from diverse Latino national origins. The tendency to consider Latinos as a homogenous or monolithic ethnic group makes any analysis of this population particularly challenging. Our finding of loci whose methylation patterns differed between Latino ethnic subgroups, even after adjusting for genetic ancestry, suggests that any analysis of these populations in disease-association studies without adjusting for ethnic heterogeneity is likely to result in spurious associations even after controlling for genomic ancestry.

In summary, this study provides a framework for understanding how genetic, social and environmental factors can contribute to systematic differences in methylation patterns between ethnic subgroups, even between presumably closely related populations such as Puerto Ricans and Mexicans. Methylation QTL's whose allele frequency varies by ancestry lead to an association between local ancestry and methylation level. This, in turn, leads to systematic variation in methylation patterns by ancestry, which then contributes to ethnic differences in genome-wide patterns of methylation. However, although genetic ancestry has been used to adjust for confounding in genetic studies, and can account for much of the ethnic differences in methylation in this study, ethnic identity is associated with methylation beyond the effects of shared genetic ancestry. This is likely due to social and environmental effects captured by ethnicity. Indeed, we find that CpG sites known to be influenced by social and environmental exposures are also differentially methylated between ethnic subgroups. These findings called attention to a more complete understanding of the effect of social and environmental variables on methylation in the context of race and ethnicity to fully understanding this complex process.

Our findings have important implications for the independent and joint effects of race, ethnicity, and genetic ancestry in biomedical research and clinical practice, especially in studies conducted in diverse or admixed populations. Our conclusions may be generalizable to any population that is racially mixed such as those from South Africa, India, and Brazil, though we would encourage further study in diverse populations, and likely has implications for all studies of diverse populations. As the National Institutes of Health (NIH) embarks on a precision medicine initiative, this research underscores the importance of including diverse populations and studying factors capturing the influence of social, cultural, and environmental factors, in addition to genetic ones, upon disparities in disease and drug response.

Materials and Methods

Participant Recruitment.

All research on human subjects was approved by the Institutional Review Board at the University of California and each of the recruitment sites (Kaiser Permanente Northern California, Children's Hospital Oakland, Northwestern University, Children's Memorial

357 Hospital Chicago, Baylor College of Medicine on behalf of the Texas Children's Hospital, VA
358 Medical Center in Puerto Rico, the Albert Einstein College of Medicine on behalf of the Jacobi
359 Medical Center in New York and the Western Review Board on behalf of the Centro de
360 Neumologia Pediatrica), and all participants/parents provided age-appropriate written
361 assent/consent. Latino children were enrolled as a part of the ongoing GALA II case-control
362 study[33].

363 A total of 4,702 children (2,374 participants with asthma and 2,328 healthy controls) were
364 recruited from five centers (Chicago, Bronx, Houston, San Francisco Bay Area, and Puerto Rico)
365 using a combination of community- and clinic-based recruitment. Participants were eligible if
366 they were 8-21 years of age and self-identified as a specific Latino ethnicity and had four Latino
367 grandparents. Asthma cases were defined as participants with a history of physician diagnosed
368 asthma and the presence of two or more symptoms of coughing, wheezing, or shortness of
369 breath in the 2 years preceding enrollment. Participants were excluded if they reported any of
370 the following: (1) 10 or more pack-years of smoking; (2) any smoking within 1 year of
371 recruitment date; (3) history of lung diseases other than asthma (cases) or chronic illness
372 (cases and controls); or (4) pregnancy in the third trimester. Further details of recruitment are
373 described elsewhere[33]. Latino sub-ethnicity was determined by self-identification and the
374 ethnicity of the their four grandparents. Due to small numbers, ethnicities other than Puerto
375 Rican and Mexican were collapsed into a single category, "other Latino". Participants whose
376 four grandparents were of discordant ethnicity were considered to be of "mixed Latino"
377 ethnicity.

378 Trained interviewers, proficient in both English and Spanish, administered questionnaires to
379 gather baseline demographic data, as well as information on general health, asthma status,
380 acculturation, social, and environmental exposures.

381 Methylation

382 Genomic DNA (gDNA) was extracted from whole blood using Wizard® Genomic DNA
383 Purification Kits (Promega, Fitchburg, WI). A subset of 573 participants (311 cases with asthma
384 and 262 healthy controls) was selected for methylation. Methylation was measured using the
385 Infinium HumanMethylation450 BeadChip (Illumina, Inc., San Diego, CA) following the
386 manufacturer's instructions.

387 1 µg of gDNA was bisulfite-converted using the Zymo EZ DNA Methylation Kit™ (Zymo
388 research, Irvine, CA) according to the manufacturer's instructions. Bisulfite converted DNA
389 was isothermally amplified overnight, enzymatically fragmented, precipitated, and re-
390 suspended in hybridization buffer. The fragmented, re-suspended DNA samples were
391 dispensed onto Infinitum HumanMethylation450 BeadChips and incubated overnight in an
392 Illumina hybridization oven. Following hybridization, free DNA was washed away, and the
393 BeadChips were extended through single nucleotide extensions with fluorescent labels. The
394 BeadChips were imaged using an Illumina iScan system, and processed using the Illumina
395 GenomeStudio Software.

396 Failed probes were identified using detection p-values using Illumina's recommendations.
397 Probes on sex chromosomes and those known to contain genetic polymorphisms in the probe
398 sequence were also excluded, leaving 321,503 probes for analysis. Raw data were normalized
399 using Illumina's control probe scaling procedure. Beta values of methylation (ranging from 0
400 to 1) were converted to M-values via a logit transformation[44].

401 Genotyping

402 Details of genotyping and quality control procedures for single nucleotide polymorphisms
403 (SNPs) and individuals have been described elsewhere[45]. Briefly, participants were
404 genotyped at 818,154 SNPs on the Axiom® Genome-Wide LAT 1, World Array 4 (Affymetrix,
405 Santa Clara, CA)[46]. We removed SNPs with >5% missing data and failing platform-specific SNP
406 quality criteria (n=63,328), along with those out of Hardy-Weinberg equilibrium (n=1845; p<10-
407 6) within their respective populations (Puerto Rican, Mexican, and other Latino), as well as
408 non-autosomal SNPs. Subjects were filtered based on 95% call rates and sex discrepancies,
409 identity by descent and standard Affymetrix Axiom metrics. The total number of participants
410 passing QC was 3,804 (1,902 asthmatic cases, 1,902 healthy controls), and the total number of
411 SNPs passing QC was 747,129. The number of participants with both methylation and
412 genotyping data was 524.

413 Ancestry and PCA analysis

414 GALA II participants were combined with ancestral data from 1000 Genomes European (CEU)
415 and African (YRI) populations and 71 Native American (NAM) samples genotyped on the

416 Axiom® Genome-Wide LAT 1 array. A final sample of 568,037 autosomal SNPs with relevant
417 ancestral data was used to estimate local and global ancestry. Global ancestry was estimated
418 using the program ADMIXTURE[47], with a three population model. Local ancestry at all
419 positions across the genome was estimated using the program LAMP-LD[48], assuming three
420 ancestral populations.

421 Principal components for the genetic data were determined using the program
422 EIGENSTRAT[40].

423 Statistical analysis

424 Unless otherwise noted, all regression models were adjusted for case status, age, sex, estimated
425 cell counts, and plate and position. To account for possible heterogeneity in the cell type
426 makeup of whole blood we inferred white cell counts using the method by Houseman et al[35].
427 Indicator variables were used to code categorical variables with more than two categories,
428 such as ethnicity. In these cases, a nested analysis of variance (ANOVA) was used to compare
429 models with and without the variables to obtain an omnibus p-value for the association
430 between the categorical variable and the outcome. For analyses of dependent beta-distributed
431 variables (such as African, European, and Native American ancestries), or cell proportion, k-1
432 variables were included in the analysis, and a nested analysis of variance (ANOVA) was used to
433 compare models with and without the variables to obtain an k-1 degree of freedom omnibus p-
434 value for the association between predictor (such as ancestry) and the outcome variable.

435 The Bonferroni method was used to adjust for multiple comparisons. For methylome-wide
436 associations, the significance threshold was adjusted for 321,503 probes, resulting in a
437 Bonferroni threshold of 1.6×10^{-7}. Analyses were performed using R version 3.2.1 (The R
438 Foundation for Statistical Computing)[49] and the Bioconductor package version 2.13.

439 Multidimensional scaling of the logit transformed methylation data (M-values) was performed
440 by first calculating the Euclidian distance matrix between each pair of individuals and then
441 calculating the first 10 principal coordinates of the data [Figure S2A]. We performed both a
442 simple correlation analysis of these principal coordinates to demographic factors (age, sex,
443 ethnicity), estimated cell counts and technical factors (batch, plate, and position) to identify
444 factors that correlated with global methylation patterns [see Figure S2B]. In addition, we

445 performed a multiple regression analysis of methylation principal coordinates by ethnicity
446 and ancestry, adjusting for case status, age, sex, estimated cell counts, and plate and position
447 [Table S1].

448 We also sought to establish the extent to which global differences in methylation between
449 Puerto Ricans and Mexicans could be explained by differences in ancestry between the two
450 groups. We estimated the proportion of the ethnicity association that was mediated by
451 genomic ancestry using the R package "mediation"[36] for methylation principal coordinates,
452 which demonstrated a significant association with ethnicity.

453 We also sought to correlate ethnicity and methylation at a locus-specific level. We thus
454 performed a linear regression between methylation at each CpG site and self-reported
455 ethnicity (Mexican, Puerto Rican, Mixed Latino, and Other Latino), followed by a three degree
456 of freedom analysis of variance to determine the overall effect of ethnicity on methylation We
457 repeated the analysis excluding the 16 participants that were self-described as "Mixed Latino",
458 and tested for non-linearity in two ways: by adding second and third order polynomials to the
459 model, and by adding a 3-degree of freedom cubic spline and comparing models with the non-
460 linear terms to those without using a nested ANOVA. At loci where there was evidence for
461 non-linearity, we tested whether ethnicity remained associated with methylation after
462 adjusting for ancestry as well as the deviations from linearity. Finally, we tested for the
463 presence of population sub-structure beyond that conveyed through ancestry by adding the
464 genetic principal components 3-10 (PCs 1 and 2 were co-linear with ancestry with a correlation
465 coefficient R2 > 0.998) and comparing models with those PCs to those without. At loci where
466 there was evidence for association between PC's 3-10 and methylation, we tested whether
467 ethnicity remained associated with methylation after adjusting for ancestry as well as the PC's
468 3-10.

469 We calculated the proportion of variance in methylation explained by ethnicity and genomic
470 ancestry at each site where ethnicity was significantly associated with methylation. To do this,
471 we fit a model that included both ethnicity and global ancestry as well as the confounders
472 described above and calculated the proportion of variance explained by multiplying the ratio
473 of the variance between predictors (ethnicity and genomic ancestry) and outcome
474 (methylation) by the square of the effect magnitude (ß).

We also examined whether differences in methylation patterns by ethnicity could be associated with known loci that had previously been reported to vary based on common environmental exposures, including maternal smoking during pregnancy[23], diesel exhaust particles (DEP)[25], and exposure to violence[27]. We have previously shown that exposure to these common environmental exposures or similar exposures varied by ethnicity within our own GALA II study populations[33,50,51].

In addition, we examined the association between global ancestry and methylation across all CpG loci using a two-degree of freedom likelihood ratio test as well as by examining the association between individual ancestral components (African, European, and Native American) and methylation at each CpG site. At each site where methylation was significantly associated with genomic ancestry proportions, we determined the relative effect of global ancestry (θ, theta) and local ancestry (γ, gamma) in a joint model by calculating the proportion of variance explained as above.

To determine whether ancestry associations with methylation were due to variation in local ancestry, we correlated local ancestry at each CpG site with methylation at the site. Because ancestry LD is much stronger than genotypic LD, it is possible to accurately interpolate ancestry at each CpG site based on the ancestry estimated at the nearest SNPs[45,52]. Measures of locus-specific ancestry were correlated with local methylation using linear regression. We performed a two-degree of freedom analysis of variance test evaluating the overall effect of all three ancestries as well as single-ancestry associations comparing methylation at a given locus with the number of African, European and Native American chromosomes at that CpG site.

Acknowledgements

The authors acknowledge the families and patients for their participation and thank the numerous health care providers and community clinics for their support and participation in GALA II. In particular, the authors thank study coordinator Sandra Salazar; the recruiters who obtained the data: Duanny Alva, MD, Gaby Ayala-Rodriguez, Lisa Caine, Elizabeth Castellanos, Jaime Colon, Denise DeJesus, Blanca Lopez , Brenda Lopez, MD, Louis Martos, Vivian Medina, Juana Olivo, Mario Peralta, Esther Pomares, MD, Jihan Quraishi, Johanna Rodriguez, Shahdad Saeedi, Dean Soto, Ana Taveras. We also thank Sasha Gusev for helpful discussion.

504 Computations in this manuscript were performed using the UCSF Biostatistics High
505 Performance Computing System.

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671

672 ## Tables

673 **Table 1**: Baseline characteristics of GALA II participants with methylation data,
674 stratified by ethnicity.

	Mexican	*Puerto Rican*	*Mixed Latino*	*Other Latino*
n	276	220	16	61
Males (%)	125 (45.3%)	127 (57.7%)	6 (37.5%)	28 (45.9%)
Age	11.4 [9.3: 14.7]	12.3 [10.4: 14.2]	11.8 [10.7: 14.9]	11.8 [10: 15.7]
Asthma cases (%)	124 (44.9%)	147 (66.8%)	9 (56.3%)	31 (50.8%)
	Ancestry (n = 524)			
African	4.3% [2.9%: 6.0%]	22.8% [16.6%: 29.4%]	8.5% [5.6%: 19.2%]	12.3% [6.3%: 25.8%]
Native American	55.4% [44.5%: 65.7%]	11.2% [9.8%: 13%]	31.5% [20.9%: 45.6%]	32.8% [10.4%: 49.3%]
European	40.5% [29.9%: 50.2%]	65.7% [59.2%: 71%]	50.5% [44.6%: 57.6%]	48.9% [40%: 58.5%]
	Recruitment Site			
Chicago	140 (50.7%)	15 (6.8%)	11 (68.9%)	15 (24.6%)
New York	18 (6.5%)	10 (4.5%)	1 (6.3%)	23 (37.7%)
Puerto Rico	0	193 (87.7%)	0	0
San Francisco	78 (28.3%)	0	2 (12.5%)	23 (37.7%)
Houston	40 (14.5%)	2 (0.9%)	2 (12.5%)	5 (8.2%)
	Cell Counts (estimated)			
Granulocytes	51.2% [46.0%: 55.7%]	51.6% [46.8%: 57%]	51% [43.6%: 57.2%]	49.1% [43.8%: 55.8%]
Lymphocytes	41.9% [36.9%: 46.6%]	41.8% [36.9%: 46.5%]	41.9% [36.1%: 51.6%]	43.9% [36.8%: 49.6%]
Monocytes	7.1% [5.8%: 8.3%]	6.74% [5.74%: 8.24%]	6.6% [5.7%: 7.6%]	7.4% [6.2%: 8.6%]

675

676 ## Figure Legends

677 **Figure 1**: Associations between ethnicity and methylation (**A**) Manhattan plot showing
678 the associations between ethnicity and methylation at individual CpG loci. (**B**) Violin
679 plot showing one such locus, cg19145607. Mexicans are relatively hypermethylated
680 compared to Puerto Ricans ($p = 1.4\times10^{-19}$). (**C**) Plot showing the association between
681 Native American ancestry at the locus and methylation levels at the locus colored by
682 ethnicity; Native American ancestry accounts for 58% of the association between
683 ethnicity and methylation at the locus.

684 **Figure 2:** Association between local ancestry and methylation. Indicate figure parts
685 with bold capital letters (**A**) Association between cg04922029 on the *DARC* locus and
686 African ancestry, color coded by ethnic group. There is near perfect correlation between
687 the two. (**B**) Association between SNPs located within 1Mb of cg04922029 and
688 methylation levels at that CpG. (**C**) Association between rs2814778 (Duffy null)
689 genotype and methylation at cg04922029, color coded by the number of African alleles
690 present. There is near perfect correlation between genotype, ancestry and methylation at
691 the locus. (**D**) Allele frequency of rs2814778 by 1000 Genomes population. The C allele
692 is nearly ubiquitous in African populations and nearly absent outside of African
693 populations and their descendants.

Figure 1

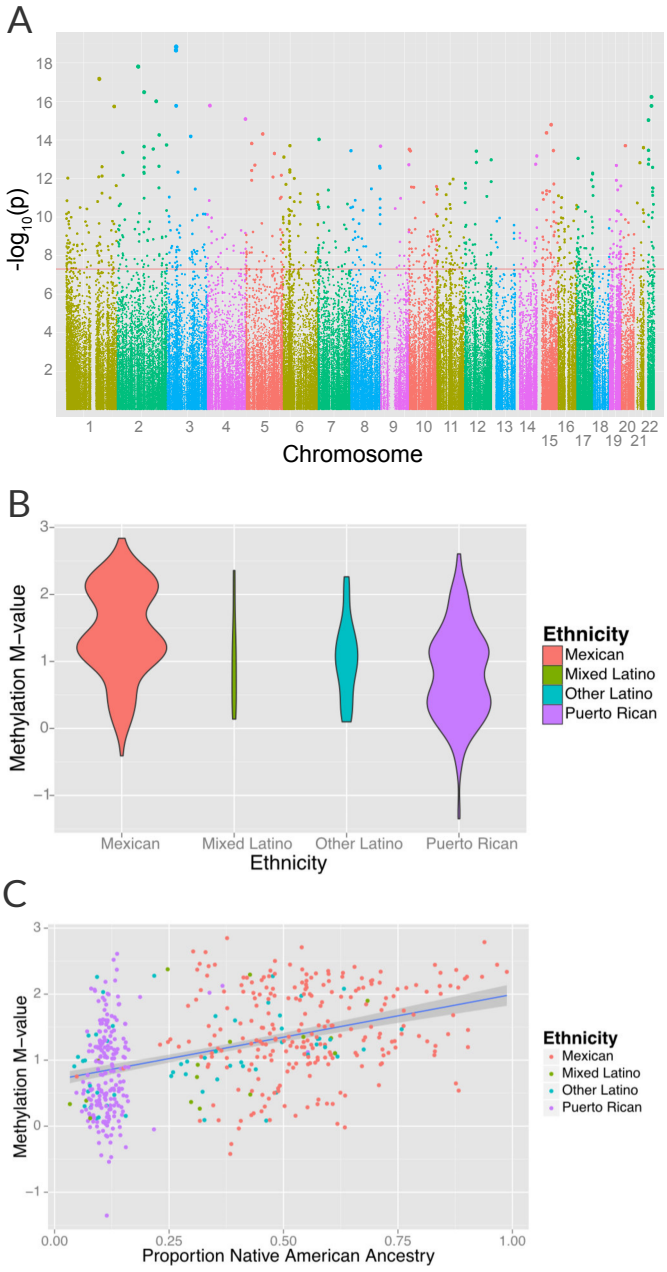
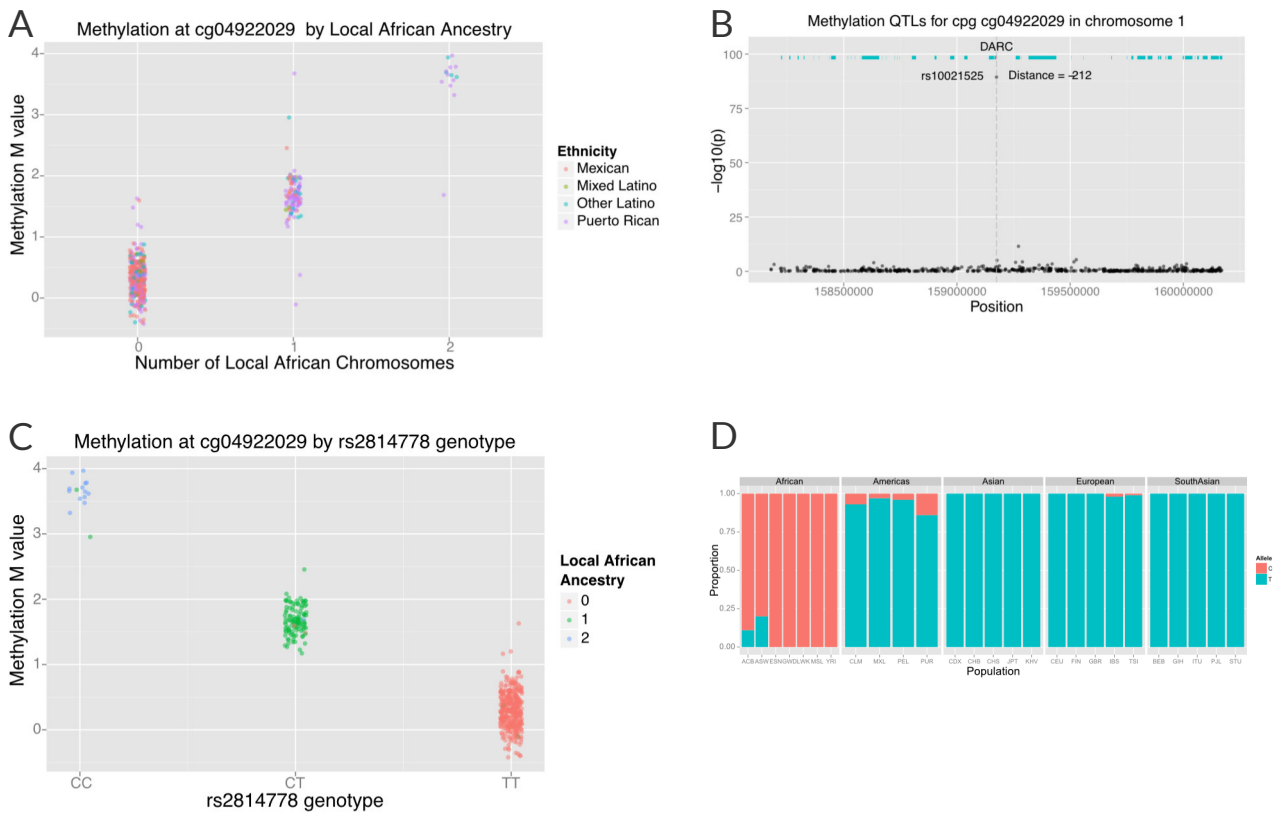

A
-log10(p)
Chromosome
B
Methylation M−value
Ethnicity
Mexican
Mixed Latino
Other Latino
Puerto Rican
C
Methylation M−value
Proportion Native American Ancestry
Ethnicity
Mexican
Mixed Latino
Other Latino
Puerto Rican

Figure 2


A
Methylation at cg04922029 by Local African Ancestry
Methylation M value
Number of Local African Chromosomes
Ethnicity
Mexican
Mixed Latino
Other Latino
Puerto Rican
B
Methylation QTLs for cpg cg04922029 in chromosome 1
DARC
rs10021525
Distance = -212
−log10(p)
Position
C
Methylation at cg04922029 by rs2814778 genotype
Methylation M value
rs2814778 genotype
CC
CT
TT
Local African Ancestry
0
1
2
D
African
Americas
Asian
European
SouthAsian
Proportion
Population
Allele
C
T