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Dynamics of Methylation of CpG Sites Associated With Systemic Lupus Erythematosus Subtypes in a Longitudinal Cohort

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Objective. Findings from cross-sectional studies have revealed associations between DNA methylation and systemic lupus erythematosus (SLE) outcomes. This study was undertaken to investigate the dynamics of DNA methylation by examining participants from an SLE longitudinal cohort using samples collected at 2 time points.

Methods. A total of 101 participants from the California Lupus Epidemiology Study were included in our analysis. DNA was extracted from blood samples collected at the time of enrolment in the cohort and samples collected after 2 years and was analyzed using Illumina EPIC BeadChip kit. Paired *t*-tests were used to identify genome-wide changes which included 256 CpG sites previously found to be associated with SLE subtypes. Linear mixed models were developed to understand the relationship between DNA methylation and disease activity, medication use, and sample cell-type proportions, adjusted for age, sex, and genetic principal components.

Results. The majority of CpGs that were previously determined to be associated with SLE subtypes remained stable over 2 years (185 CpGs [72.3%]; *t*-test false discovery rate >0.05). Compared to background genome-wide methylation, there was an enrichment of SLE subtype–associated CpGs that changed over time (27.7% versus 0.34%). Changes in cell-type proportions were associated with changes at 67 CpGs ($P < 2.70 \times 10^{-5}$), and 15 CpGs had at least 1 significant association with immunosuppressant use.

Conclusion. In this longitudinal SLE cohort, we identified a subset of SLE subtype–associated CpGs that remained stable over time and may be useful as biomarkers of disease subtypes. Another subset of SLE subtype–associated CpGs changed at a higher proportion compared to the genome-wide methylome. Additional studies are needed to understand the etiology and impact of these changes on methylation of SLE-associated CpGs.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease that affects 1 in 600 women in the US, and it is among the leading causes of death in young women, despite modern treatments (1,2). Prior studies suggest that epigenetics informs SLE disease heterogeneity and pathophysiology. Epigenetics is the study of chromatin modifications, including DNA methylation, that regulate gene expression and cell differentiation (3). Changes in methylation of CpG sites within interferon-responsive genes and regulatory regions of the genome and in different immune cell types are associated with SLE risk, disease activity, and specific organ manifestations such as lupus nephritis (4–9). However, causality of these associations cannot be determined given the cross-sectional nature of previous studies. Furthermore, the stability of CpG

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methylation changes and the prognostic implications for long-term outcomes remain unclear.

There are many challenges in treating SLE, and there is a lack of available biomarkers that can be used to accurately predict clinical outcomes and response to treatment. DNA methylation in whole blood samples is an attractive biomarker, as samples can be easily obtained and do not require the isolation of peripheral blood mononuclear cells (PBMCs) or cell sorting. Thus, whole blood DNA methylation has potential to be easily applied in clinical practice as a tool for precision medicine. Therefore, understanding the longitudinal stability and variability of the methylome in SLE patients is fundamental to its utility as a biomarker.

In this study, we investigated the longitudinal trajectory of DNA methylation in whole blood samples from a diverse multiethnic cohort of SLE patients followed up for over 2 years. We had previously performed an epigenome-wide association study (EWAS) of all participants from the California Lupus Epidemiology Study (CLUES) at cohort enrolment (10). We identified 3 patient subtypes at enrolment in the cohort according to American College of Rheumatology (ACR) classification criteria and subcriteria (11,12). We classified the patients as mild (M), severe 1 (S1), and severe 2 (S2) according to autoantibody pattern and internal organ involvement and identified 256 CpGs that were significantly associated with these subtypes, many of which mapped to the interferon pathway.

Here, we examined the dynamics of this previously described DNA methylation signature as well as the genome-wide longitudinal trajectory of the methylome in participants from the CLUES cohort (n = 101). We studied the impact of disease activity, medication use, cell-type proportions, genetic variation, and self-reported ethnicity and race on changes in methylation at CpG sites.

PATIENTS AND METHODS

Subjects. CLUES is a multiracial and multiethnic cohort of individuals with physician-confirmed SLE. This study was approved by the Institutional Review Board of the University of California, San Francisco. Written informed consent was obtained from all patients prior to participation in the study. Participants were recruited from the California Lupus Surveillance Project, a population-based cohort of individuals with SLE living in the County of San Francisco from 2007 to 2009 (13). Additional participants residing in the geographic region were recruited through local academic and community rheumatology clinics and through existing local research cohorts. This study included a subset of 101 CLUES participants from the following self-reported races and ethnicities: White (n = 29), Black (n = 13), Asian (n = 34), Hispanic (n = 22), and other or unspecified (n = 3). Clinical and demographic characteristics are shown in Supplementary Table 1 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.42237).

Study procedures involved an in-person research clinic visit every 2 years including the collection and review of medical records prior to each visit, a history of SLE and physical examination conducted by a physician specializing in lupus, collection of biologic specimens including peripheral blood samples for clinical and research purposes, and completion of a structured interview administered by an experienced research assistant. For the 101 participants, the mean ± SD time between visits was 2.3 ± 0.3 years. All SLE diagnoses were confirmed by study physicians (CML, MD, and JY) according to 1 of the following definitions: 1) meeting ≥4 of 11 ACR revised criteria for the classification of SLE as defined in 1982 (11) and updated in 1997 (12), 2) meeting 3 of 11 ACR criteria and having a documented rheumatologist's diagnosis of SLE, or 3) a rheumatologist-confirmed diagnosis of lupus nephritis, defined as having evidence of lupus nephritis on kidney biopsy. Medication use at the time of blood collection was recorded. For data analyses, we grouped immunosuppressive medications into the following categories: biologic treatments (belimumab, abatacept, rituximab), low-dose prednisone (<10 mg), moderate or high-dose prednisone (>10 mg), antimalarials, calcineurin inhibitors, methotrexate and leflunomide, azathioprine, mycophenolate mofetil, and cyclophosphamide. Self-reported race and ethnicity data was collected from each study participant.

DNA methylation and quality control. Methylation of genomic DNA from whole blood samples was profiled using an Illumina Methylation EPIC BeadChip kit. This chip kit assesses the methylation level of ~850,000 CpGs in enhancer regions, gene bodies, promoters, and CpG islands. All subsequent processing was conducted using the R minfi package. Signal intensities were background subtracted using the minfi noob function and were then quantile normalized (14,15). We removed sites with a poor detection rate ($P > 0.05$) in more than 5% of the samples (1,746 CpG sites). We removed sites where probes were predicted to hybridize to multiple loci (44,097) and sites on nonautosomal chromosomes (19,627 CpG sites). We also excluded 91,799 CpGs that have been shown to poorly perform due to single-nucleotide polymorphisms (SNPs) near probes in diverse populations (16). Additionally, we removed 3,413 CpGs where the assay control sample had a variance >0.01 across the 9 plates. After implementing these quality control measures, 720,682 CpGs were included in the analysis.

DNA genotyping. Genotyping of genomic DNA from blood samples was performed using Affymetrix Axiom Genome-Wide LAT 1 Array. This genotyping array is composed of 817,810 SNP markers across the genome and was specifically designed to provide maximal coverage of diverse racial and ethnic populations, including West Africans, Europeans, and American Indians (17). Samples were retained with Dish QC ≥0.82. SNP genotypes were first filtered for high-quality cluster differentiation and 95% call rate within batches using SNPolisher. Additional quality

control was performed using Plink. SNPs with an overall call rate <95% or discordant calls in duplicate samples were removed. Samples were excluded if there were unexpected duplicates in identity by descent analysis or if the sex was mismatched between genetic data and self report; 1 sample was retained for first-degree relatives. All samples had at least 95% genotyping and no evidence of excess heterozygosity (maximum <2.5*SD). We tested for Hardy-Weinberg equilibrium and cross-batch association for batch effects using a subset of subjects that were of European ancestry and were negative for double-stranded DNA (dsDNA) antibodies and renal disease to minimize genetic heterogeneity. SNPs were excluded if Hardy-Weinberg equilibrium $P < 1 \times 10^{-5}$ or any cross-batch association $P < 5 \times 10^{-8}$. Genetic principal components were calculated to account for population structure using PCAmixdata R package.

Genetic ancestry. We performed ADMIXTURE [18] analysis using genome-wide SNP data to estimate the percent contribution of each ancestral population for each participant in the study. We first combined our sample data with 1000 Genomes genotype data and removed SNPs for linkage disequilibrium according to software recommendations, excluding each SNP with an $R^2 > 0.1$ in a 50-SNP sliding window advanced by 10 SNPs each time. After exclusion, 162,159 SNPs were used to estimate global ancestry. We then ran ADMIXTURE unsupervised assuming 5 subpopulations (European, African, East Asian, South Asian, and American Indian). We then used labels from 1000 Genomes to determine the ancestry of the estimated proportions of each of our subjects for downstream analysis.

Differential methylation analysis. Our analysis pipeline is shown in Figure 1. Samples from different time points were quantile normalized together. Principal components analysis plots between different time points, plates, and race and ethnicities are shown (Supplementary Figures 1–3, http://onlinelibrary. wiley.com/doi/10.1002/art.42237). Significance testing was performed using M values, with effect sizes converted to beta values for reporting. To adjust for differences between plates, we used ComBat [19]. We adjusted the beta values with residual values for estimated cell-type proportions using the reference-based Houseman algorithm to account for cell-type proportion heterogeneity [20,21]. We initially performed a genome-wide paired

The California Lupus Epidemiology Study (CLUES)
Racially and ethnically diverse longitudinal cohort of over 332 deeply phenotyped SLE patients with DNA methylation at cohort enrollment

101 patients were selected from the CLUES cohort

DNA methylation on whole blood at cohort enrollment and at a 2 year follow up

Quality Control: Total CpGs retained for analysis =720,682

Adjusted matrix with residuals for cell proportions

Unadjusted matrix

Paired t-test between DNA methylation at each time point
Significant CpGs selected: FDR<0.05 and |β|>0.03

Examined significant CpGs from paired t-test

Significant CpGs that changed over time were examined in repeated measures mixed linear models adjusting for age, genetic PCs and sex to explore the effects of:
a) SLEDAI score, dsDNA positivity and lupus nephritis
b) medications

glm models with change in methylation as a function of change in cell proportions, adjusting for age, sex and genetic PCs

Figure 1. Analysis pipeline of our study of 101 participants from the CLUES study. SLE = systemic lupus erythematosus; FDR = false discovery rate; PCs = principal components; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; dsDNA = double-stranded DNA; glm = generalized linear model. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42237/abstract.

t-test of the 2 time points and retained CpG sites that had a false discovery rate (FDR) $P < 0.05$ and an absolute beta value difference of >0.03, as a threshold for an effect size to be biologically meaningful ([22]).

We then took a closer look at the previously described SLE subtype–associated CpGs within the genome-wide results. We constructed linear mixed models to analyze repeated measurements with DNA methylation as the outcome to investigate the effect of disease activity, dsDNA titer at the time of blood was drawn, lupus nephritis status, and medication use adjusted for sex, age, and genetic principal components (principal components 1–3). To examine the role of cell-type proportion heterogeneity, significant CpGs from the paired *t*-tests were reanalyzed without adjusting for cell-type proportions. Changes in DNA methylation was modeled with the change in each cell-type proportion as a predictor, adjusting for sex, age, and genetic principal components (principal components 1–3). All association analyses were performed using R version 3.6 and Stata 13.1. Pathway analysis was performed using ToppFun ([23]).

SLE subtype–associated CpGs enrichment analysis.
Enrichment of SLE subtype–associated CpGs in CpGs with a significant change over time was determined via the following methods. Briefly, we determined the methylation variance of

the 256 CpGs that were associated with SLE subtypes at cohort enrolment. Then, randomly selected 256 CpG sites with similar methylation variance distribution compared to SLE subtype–associated CpGs throughout the genome were tested to see if there was any difference in methylation at the 2 time points (paired *t*-test). We tested this for a total of 100 random samples. Results were compared to CpGs associated with SLE subtypes.

Statistical methylation quantitative trait loci (QTLs) analysis.
We previously reported methylation QTL analysis findings on SLE subtype–associated CpGs at the time of enrolment in the cohort ([10]). Briefly, this was performed by fitting a linear model adjusted for sex, age, cell-type proportions, alcohol use, smoking status, the top 3 genetic principal components, and the top 3 medication principal components using the Matrix eQTL R package ([24]). There are also larger established data sets of CpGs in healthy individuals that provide evidence of genetic control ([25]). Combining our own findings and available resources, we identified a total of 39,899 CpGs with evidence of methylation QTL within the Illumina Methylation EPIC BeadChip kit that passed our quality control. We used a 2-sample test to see if the proportion of methylation QTLs in CpGs that had a significant change over time was higher than the proportion of methylation QTLs in stable CpGs.

Table 1. Selected candidate CpGs that were stable over time and had the greatest difference in methylation between SLE clinical subtypes at the time of enrollment in the CLUES cohort*

| CpG | Gene | CpG position | Mean methylation beta values at enrollment, by cluster | | | Variance‡ | IFNα-responsive gene | IFNγ-responsive gene |
			Mild†	Severe 1†	Severe 2†			
cg16987437	SP100	Body	0.623	0.536	0.481	0.0178	No	No
cg15065340	TNK2	5′-UTR	0.623	0.555	0.499	0.0152	No	No
cg19188021	ODF3B	5′-UTR	0.264	0.174	0.142	0.0142	No	No
cg17114584	IRF7	Body	0.513	0.451	0.399	0.0137	Yes	Yes
cg22012079	IFI44L	5′-UTR	0.586	0.501	0.462	0.0126	Yes	Yes
cg12461141	TRIM22	TSS1500	0.493	0.423	0.380	0.0115	No	No
cg14333162	RSAD2	TSS1500	0.698	0.647	0.602	0.0092	Yes	Yes
cg26531432	RABGAP1L	5′-UTR	0.698	0.634	0.605	0.0087	No	No
cg20343278	PTPRM	Body	0.323	0.361	0.304	0.0087	No	No
cg03540917	SPINK2	Body	0.599	0.627	0.669	0.0086	No	No
cg15378061	NA	NA	0.186	0.231	0.257	0.0084	No	No
cg15331332	HLA-F	Body	0.599	0.568	0.538	0.0081	No	No
cg00272009	PARP14	TSS1500	0.631	0.581	0.552	0.0080	Yes	Yes
cg25178683	LGALS3BP	TSS1500	0.554	0.509	0.470	0.0077	Yes	Yes
cg13045500	NA	NA	0.659	0.604	0.569	0.0072	No	No
cg06168856	OAS1	Body	0.630	0.598	0.575	0.0067	Yes	No
cg05167074	SHKBP1	Body	0.555	0.511	0.489	0.0067	No	No
cg06708931	NA	NA	0.906	0.867	0.828	0.0064	No	No
cg06650861	DDX60	5′-UTR	0.868	0.830	0.792	0.0064	Yes	Yes
cg06376949	IFIT5	TSS1500	0.255	0.210	0.177	0.0063	No	No

* Mean methylation beta values at the time of enrollment were compared by paired *t*-test, with a false discovery rate of >0.05. Cohort participants were clustered according to American College of Rheumatology systemic lupus erythematosus (SLE) classification criteria and subcriteria ([11,12]) using an unsupervised clustering approach ([10]). CLUES = California Lupus Epidemiology Study; IFN = interferon; UTR = untranslated region; TSS = transcription start site; NA = not applicable.
† Patients were classified as mild, severe 1, and severe 2 according to autoantibody pattern and internal organ involvement.
‡ By analysis of variance F test.
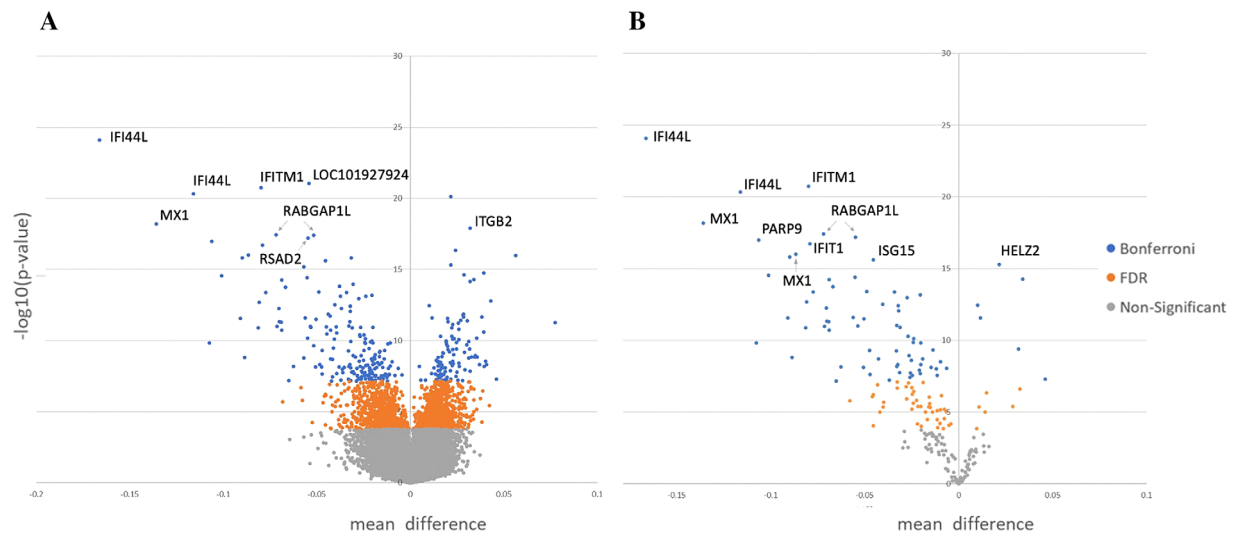
Figure 2. **A**, Systemic lupus erythematosus (SLE) subtype–associated CpGs with a significant change in methylation over the 2 time points. **B**, Methylation levels of 2,423 CpGs across the genome that significantly changed over a period of 2 years (0.34% of the represented methylome). Symbols represent individual samples. FDR = false discovery rate.
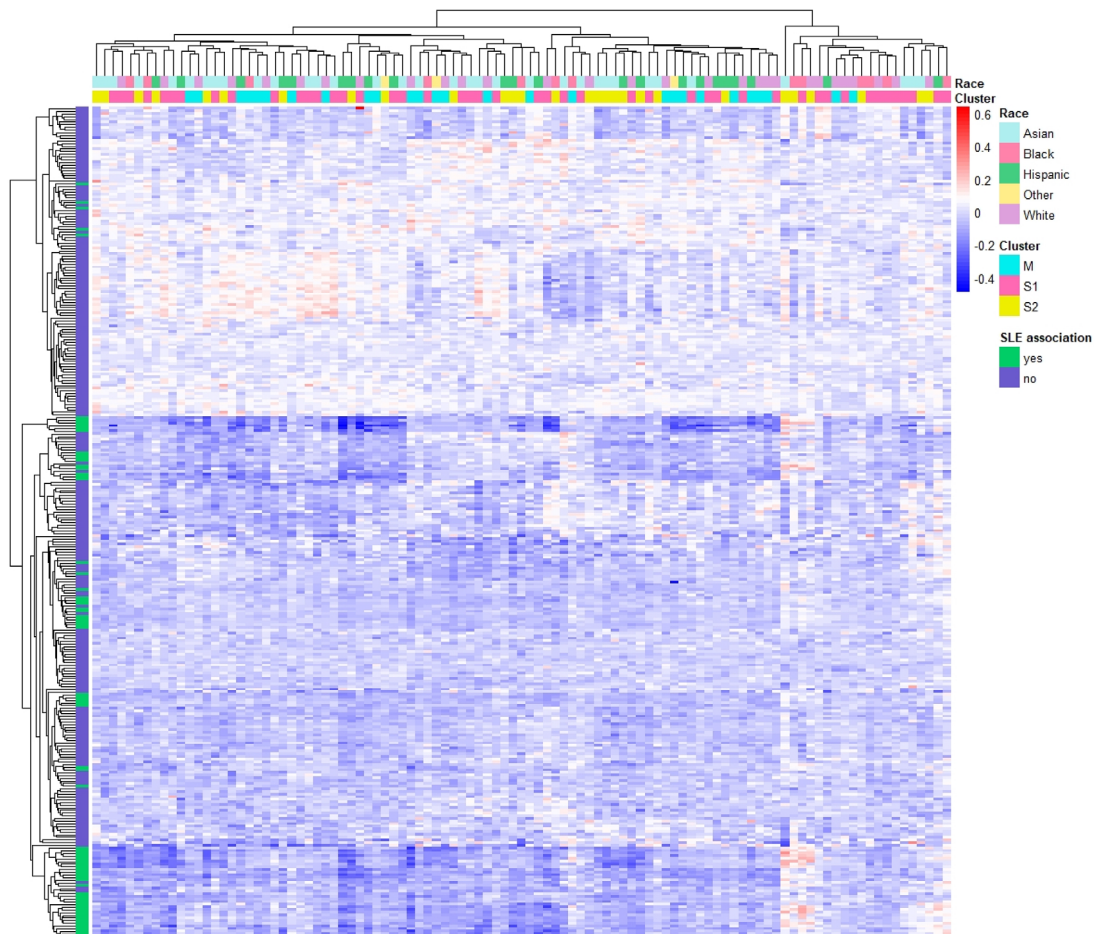
Figure 3. Heat map of CpG sites with a significant methylation change in a 2 year period. We observed 309 CpGs with a DNA methylation difference (absolute beta value difference >0.03, false disovery rate <0.05) in a 2-year time period. Each row represents a CpG and each column represents a participant with systemic lupus erythematosus (SLE).

Table 2. Top 20 CpGs whose methylation significantly changed over a 2-year time period among SLE patients in the CLUES cohort*

CpG	Gene	Mean methylation beta values, time point 1 to time point 2				Paired t-test	
		Time point 1	Time point 2	Delta	Fold change	P	FDR
cg13452062	IFI44L†	0.31	0.14	0.17	–1.17	8×10^{-25}	5.97×10^{-19}
cg07929412	LOC101927924	0.75	0.70	0.05	–0.08	9×10^{-22}	3.33×10^{-16}
cg23570810	IFITM1†	0.49	0.41	0.08	–0.20	2×10^{-21}	4.32×10^{-16}
cg05696877	IFI44L†	0.31	0.19	0.12	–0.60	5×10^{-21}	8.45×10^{-16}
cg21549285	MX1†	0.40	0.26	0.14	–0.52	7×10^{-19}	7.94×10^{-14}
cg14628347	ITGB2	0.63	0.67	0.03	0.05	1×10^{-18}	1.33×10^{-13}
cg25984164	RABGAP1L†	0.71	0.63	0.07	–0.11	4×10^{-18}	3.29×10^{-13}
cg10549986	RSAD2	0.15	0.10	0.05	–0.51	4×10^{-18}	3.29×10^{-13}
cg09948374	RABGAP1L†	0.60	0.55	0.05	–0.10	6×10^{-18}	4.65×10^{-13}
cg07815522	PARP9†	0.45	0.34	0.11	–0.31	1×10^{-17}	6.88×10^{-13}
cg05552874	IFIT1†	0.40	0.32	0.08	–0.25	2×10^{-17}	1.14×10^{-12}
cg22862003	MX1†	0.41	0.33	0.09	–0.27	1×10^{-16}	5.06×10^{-12}
cg18467790	RADIL	0.52	0.58	0.06	0.10	1×10^{-16}	5.06×10^{-12}
cg16526047	ISG15	0.49	0.46	0.03	–0.07	2×10^{-16}	6.43×10^{-12}
cg24678928	DDX60†	0.70	0.61	0.09	–0.15	2×10^{-16}	6.43×10^{-12}
cg20062691	ISG15†	0.66	0.62	0.05	–0.07	3×10^{-16}	1.01×10^{-11}
cg07469075	PAMR1	0.58	0.52	0.06	–0.11	7×10^{-16}	2.44×10^{-11}
cg11317199	TRIM14	0.59	0.63	0.04	0.06	2×10^{-15}	5.99×10^{-11}
cg08565796	HKR1	0.32	0.35	0.03	0.08	2×10^{-15}	8.09×10^{-11}
cg12439472	EPSTI1†	0.31	0.21	0.10	–0.48	3×10^{-15}	8.94×10^{-11}
cg05883128	DDX60†	0.33	0.27	0.06	–0.20	4×10^{-15}	1.19×10^{-10}
cg13100600	AGRN†	0.51	0.54	0.03	0.06	5×10^{-15}	1.52×10^{-10}
cg07839457	NLRC5†	0.24	0.17	0.07	–0.40	6×10^{-15}	1.56×10^{-10}
cg25267487	NA	0.67	0.71	0.03	0.05	7×10^{-15}	1.86×10^{-10}
cg13207212	APBB2	0.56	0.52	0.03	–0.06	1×10^{-14}	2.81×10^{-10}

* For the full list of 309 CpGs, see Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.42237. CLUES = California Lupus Epidemiology Study; FDR = false discovery rate; NA = not applicable.
† CpGs were associated with systemic lupus erythematosus (SLE) subtypes at the time of enrollment in the cohort.

RESULTS

Stability of the majority of SLE subtype–associated CpGs over time. In previous studies, CLUES cohort participants were clustered into 3 subtypes according to ACR classification criteria at the time of enrolment in the cohort. We identified 256 CpGs that were differentially methylated according to subtype (10).

In the current study, we observed the dynamics of DNA methylation in our previous findings by comparing data collected at 2 time points. Of the 256 CpGs that were associated with disease subtypes, 184 CpGs (71.9%) were stable between the 2 time points. Since we observed an enrichment of CpGs in interferon (IFN)–responsive genes in 256 CpGs, we investigated whether there was a difference in terms of dynamics between CpGs in IFN-responsive genes compared to non–IFN-responsive genes. We found that 53% of CpGs in IFN-responsive genes were stable compared to 87% of CpGs in non–IFN-responsive genes ($P = 1.4 \times 10^{-9}$ by chi-square test), indicating that CpGs in IFN-responsive genes are more susceptible to change in methylation. Regarding CpG position relative to genes, 74 were in gene bodies (40.2%), 50 were in transcription start sites (27.2%), and 30 were in untranslated regions (16.3%). Twenty CpGs with the

most variance across clinical clusters (10) that did not change over time are shown in Table 1, and the full list of stable CpGs is shown in Supplementary Table 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.42237).

These include CpGs in *TNK2, RABGAP1L, IRF7, IFI44L, TRIM22*, and many IFN-responsive genes. DNA methylation within/near these genes has been implicated in SLE in previous studies; for example *TNK2* has been implicated in renal disease in CD4+ naive cells (4) and *RABGAP1L* has been implicated with anti-dsDNA antibody production. Volcano plots of representative stable CpGs are shown in Supplementary Figure 4 (http://onlinelibrary.wiley.com/doi/10.1002/art.42237).

SLE subtype–associated CpGs compared to the genome-wide methylome over time. Although the majority of SLE subtype–associated CpGs were stable, 71 CpGs (27.7%) had a significant change in methylation (>0.03) (FDR $P < 0.05$) (Figure 2A). We also examined the dynamics of the genome-wide methylome. Paired t-test analysis revealed that the methylation level of 2,423 CpGs across the genome significantly changed over a period of 2 years (FDR <0.05), which is 0.34% of the represented methylome (Figure 2B). We also

Table 3. Pathway analysis of 309 CpG sites that showed significant methylation changes (FDR <0.05) and an absolute methylation beta value difference of >0.03 over 2 years in the CLUES cohort*

CpG site identifier	Pathway name	P	FDR†	No. of genes from input	No. of genes in annotation
M39748	Human immune response to tuberculosis	2.82×10^{-8}	1.70×10^{-5}	6	23
M39583	Novel intracellular components of RLR pathway	1.20×10^{-5}	3.60×10^{-3}	6	61
M1462	CTL-mediated immune response against target cells	1.59×10^{-4}	0.023	3	13
M39909	Host–pathogen interaction of human coronaviruses–IFN induction	1.64×10^{-4}	0.023	4	33
M22023	Antigen processing and presentation	2.01×10^{-4}	0.023	3	14
M39363	Type II IFN signaling (*IFNG*)	2.58×10^{-4}	0.023	4	37
M40067	SARS–CoV-2 innate immunity evasion and cell-specific immune response	2.68×10^{-4}	0.023	5	68
M15913	RLR signaling pathway	3.28×10^{-4}	0.024	5	71
M39837	Cytosolic DNA-sensing pathway	3.98×10^{-4}	0.025	5	74
MAP00430‡	MAP00430 taurine and hypotaurine metabolism	4.22×10^{-4}	0.025	2	4
M19708	Type II diabetes mellitus	6.53×10^{-4}	0.036	4	47
M39543	Structural pathway of IL-1	8.27×10^{-4}	0.042	4	50

* Pathway analysis was performed using ToppFun (23). Unless otherwise indicated, the source of each CpG site was Molecular Signatures Database C2 BioCarta (version 7.3). CLUES = California Lupus Epidemiology Study; RLR = retinoic acid–inducible gene 1–like receptor; CTL = cytotoxic T lymphocyte; IFN = interferon; IL-1 = interleukin-1.
† Benjamini and Hochberg false discovery rate (FDR).
‡ GenMAPP was the source of this CpG site.

filtered results using a minimum difference in DNA methylation (absolute beta value difference >0.03) and observed that 309 CpG sites had the minimum difference and FDR (Figure 3 and Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.42237).

These CpGs were distributed across the genome with the top results within or near *IFI44L, IFIT1, LOC101927924*, and *MX1*. The top 25 results according to smallest *P* value are shown in Table 2. Pathway analysis of the genes containing these 309 sites identified the human immune response to tuberculosis and retinoic acid–inducible gene 1–like receptor pathways as the most significant pathways; however, multiple immune pathways were represented, including antigen processing, virus response, type II IFN signaling, cytotoxic T lymphocyte–associated pathways, and taurine and hypotaurine metabolism (Table 3). Across S1, M1, and M2 clinical subtypes, we found no significant difference in terms of the change in methylation within changing CpGs was identified between the 3 subtypes (FDR >0.05 by analysis of variance).

In comparison to the genome-wide results, there was strong evidence of enrichment in SLE subtype–associated CpGs that changed over time (27% versus 0.34%, $P = 1.82 \times 10^{-175}$). These included CpGs in *IFI44L, MX1*, and *PRABCAP1L*. A total of 68 of these 71 CpGs had a decrease in methylation and only 3 had an increase in methylation at the second time point. An enrichment analysis was performed, with results supporting this finding. In 63 of 100 times, no CpG showed a significant difference in methylation (paired *t*-test FDR >0.05; methylation beta value difference < 0.03). In 2 samples, 3 CpGs had a significant change ($P < 7 \times 10^{-8}$; methylation beta value difference >0.03), 5 samples had 5 significant CpGs, and 30 samples 1 significant CpG. The distribution of CpGs with a significant change in the

enrichment analysis is shown (see Supplementary Figure 5, http://onlinelibrary.wiley.com/doi/10.1002/art.42237).

Association of CpG sites with clinical outcomes. Although the disease was stable or quiescent over time in most study participants, a small percentage of participants had significant changes in clinical manifestations, such as changing dsDNA titers or development of lupus nephritis. In these cases, we evaluated whether CpG sites that changed over time were associated with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score (26), dsDNA antibody positivity, and/or lupus nephritis (Supplementary Table 4, http://onlinelibrary.wiley.com/doi/10.1002/art.42237). Overall, no CpGs met the threshold of significance ($P < 1.8 \times 10^{-5}$) but, according to SLEDAI scores, some evidence of an association was observed with cg09858955 in *VRK2* (β coefficient −1.2, $P = 0.001$), cg09128104 in *RARA* (β coefficient 0.69, $P = 0.00036$), and cg21524061 in *TLR6* (β coefficient 0.45, $P = 0.0005$). These genes are involved in granulopoiesis (*RARA*) (27), apoptosis (*VRK2*) (28), and immune activation (*TLR6*) (29), all of which are pathways relevant to lupus disease pathogenesis. The top associations with regard to dsDNA positivity were cg01971407 in *IFITM1* (β coefficient −0.30, $P = 0.0003$), cg05070493 in *TRAF3* (β coefficient −0.06, $P = 0.0003$), and cg00959259 and cg08122652 in *PARP9* (β coefficient −0.029, $P = 0.0003$ and β coefficient −0.11, $P = 0.0004$, respectively). Similarly, TNF receptor–associated factor 3 is known to be a powerful negative regulator of B cell survival and activation (30), *IFITM1* is an IFN-responsive gene, and *PARP9* is associated with macrophage activation (31,32).

Effect of medications on DNA methylation. Since medications such as prednisone and methotrexate can alter the

methylome in immune cells, we examined whether the changes in methylation at CpG sites were associated with the use of particular medications in a repeated measures model, adjusting for age, sex, and genetic principal components. A total of 15 of 309 CpGs (4.9%) had at least 1 significant association with use of an immunosuppressive medication (Supplementary Table 5, http://onlinelibrary.wiley.com/doi/10.1002/art.42237). Nine CpGs correlated with prednisone, 5 CpGs correlated with mycophenolate mofetil, and 2 CpGs correlated with azathioprine. There were no significant associations with changes in biologic treatments, inhibitors of purine and pyrimidine synthesis, calcineurin inhibitors, or antimalarials.

Effect of cell-type proportions on DNA methylation. One of the limitations of using whole blood DNA methylation measures in population-based studies is that differences in methylation might be due to differences in cell-type proportions between individuals at the time blood was drawn or changes taking place in cell-type proportions between blood sample collections over time. As expected, paired comparisons between the 6 estimated cell types at the 2 time points revealed significant changes in terms of the proportions of monocytes, granulocytes, and CD8+ cells (paired t-test $P < 0.05$). In studies of SLE, overadjustment of cell-type proportion differences may lead to incorrect conclusions, since changes in cell-type proportions may be relevant to disease pathogenesis. To address these issues, we initially used the adjusted matrix of cell-type proportions to determine if there were any DNA methylation changes. Then, to examine the effects of changes in cell-type proportions on the change in methylation we used the unadjusted matrix and longitudinal models incorporating the difference in DNA methylation as the outcome and the difference in each cell type as a predictor, adjusting for age, sex, and genetic principal components. We observed that 67 CpGs of the initial 309 (21.7%) that changed over time had a significant correlation with changes in at least 1 cell type estimate ($P < 2.70 \times 10^{-5}$).

Changes in DNA methylation correlated with changes in cell-type proportions at 64 CpGs for granulocyte estimates, 39 CpGs for CD4+ T cell estimates, 24 CpGs for CD8+ T cell estimates, 5 CpGs for monocyte estimates, 12 CpGs for B cell estimates, and 6 CpGs for natural killer (NK) cell estimates. Supplementary Figure 6 shows the effect sizes of changes in DNA methylation in relation to changes in cell-type proportions (http://onlinelibrary.wiley.com/doi/10.1002/art.42237). Although the largest number of CpGs influenced by changes in cell-type proportions was in granulocytes, the largest effect sizes were observed for the NK cell estimates, for example, cg0571263 (β coefficient −20.25, $P = 5.9 \times 10^{-8}$), $IFITM1$ (cg09026253) (β coefficient −13.95, $P = 6.4 \times 10^{-7}$), and $RAB6B$ (β coefficient 14.55, $P = 1.6 \times 10^{-5}$). Studies have shown $RAB6B$ expression in NK cells as well as in mucosal-associated invariant T cells (33).

We found other interesting examples of correlations of changes in DNA methylation with the change in cell-type proportions in genes known to be enriched in a particular immune cell type. For example, methylation at $RPS6KB1$ correlated with B cell estimates (cg02095219) (β coefficient 10.01, $P = 4.839 \times 10^{-7}$), where RPS6KB1 expression is known to be enriched in treatment-naive B cells and memory B cells (34). Other examples include CD4+ T cell estimates with methylation in $B2M$ (cg03425812) (β coefficient −7.45; $P = 2.489 \times 10^{-8}$) and $IFIT1M$ (cg04582010) (β coefficient −6.40, $P = 4.540 \times 10^{-8}$), known to be widely expressed in multiple CD4+ T cell subsets (33,35). Methylation at $B36NT3$ (cg16744531) (β coefficient 5.50, $P = 0.00001$) was associated with CD8+ T cell estimates demonstrated to be expressed in CD8+ memory T cells. Methylation of $TNFSF10$ (cg10213935) (β coefficient −6.633, $P = 0.000012$) was associated with monocyte estimates, where tumor necrosis factor superfamily member 10 expression is known to be enriched in intermediate, classic, and nonclassic monocytes. Finally, methylation at $IFITM1$ (cg05552874) (β coefficient −2.93, $P = 2.136 \times 10^{-6}$) and $HDAC4$ (cg27074582) (β coefficient −1.52681, $P = 1.794 \times 10^{-9}$) was associated with granulocyte estimates.

Effect of self-reported ethnicity or race, genetic ancestry, and genetic variation on methylation changes over time. There is substantial evidence that DNA methylation differs across ethnic groups. Some of these differences are due to genetic variation and some are not explained by genetics alone (36,37). To examine the effects of genetic ancestry as well as self-reported ethnicity and race, we constructed models with the methylation difference over time as the outcome and self-reported ethnicity and race or genetic ancestry estimates as predictors, adjusting for age and sex. Nine models were generated: 1 each for the self-reported ethnicities or races Hispanic, Black, White, or Asian and 1 each for the genetic ancestry estimates African, East Asian, American Indian, South Asian, European. Results are shown in Supplementary Table 6 (https://onlinelibrary.wiley.com/doi/10.1002/art.42237). No model was significant upon multiple hypothesis testing ($P < 3.2 \times 10^{-5}$); however, there are a few associations that are worth mentioning. The top methylation change association was at cg23876832 (no gene name is associated with this methylation site), with South Asian ancestry ($P = 3.75 \times 10^{-5}$). When looking at results with a nominal P value ($P < 0.05$), we found methylation change associations in 30 CpGs correlated with African ancestry, 5 CpGs correlated with European ancestry, 12 CpGs correlated with American Indian ancestry, 13 CpGs correlated with East Asian ancestry, and 12 correlated CpGs with South Asian ancestry. There were few overlaps, with 8 CpGs associated with 2 ancestries. When examining self-reported ethnicity or race, the top methylation change association was in cg00569896 (no gene name is associated with this site), associated with Black race ($P = 1.68 \times 10^{-4}$). Results

with a nominal *P* value (*P* < 0.05) indicated methylation change associations in 23 CpGs were associated with Black race, 19 CpGs were associated with Asian race, 8 CpGs were associated with Hispanic ethnicity, and 2 CpGs were associated with White race. Similarly, there were few overlaps, with 5 CpGs significantly associated with 2 ethnic or racial groups (*P* < 0.05). In terms of the significant overlap of CpGs between ancestry and self-reported ethnicity and race (*P* < 0.05), we found that 20 CpGs were associated with Black race and African ancestry, 2 CpGs were associated with Asian race and East Asian ancestry, there was no overlap between Asian race and South Asian ancestry, 2 CpGs were associated with Hispanic ethnicity and American Indian race, and there was no overlap between European ancestry and White race. These results suggest that both ancestry and self-reported ethnicity or race may be influencing methylation changes, primarily at different sites. The only exception was the high concordance between CpGs associated with Black race and African ancestry.

To test if genetic variation influenced methylation changes at specific sites, we investigated CpGs known to be under genetic control (methylation QTLs). Of the 309 CpGs that changed over time, there were 75 CpG with evidence of methylation QTL (24%). This is in comparison to 5.5% of methylation QTLs in stable CpGs genome wide (n = 39,824), suggesting an enrichment of methylation QTLs in CpGs with a significant change in methylation over time ($P = 4.8 \times 10^{-47}$ by 2-sample test). Of the 72 SLE subtype–associated CpGs that changed over time, 24 were methylation QTLs (33%). This was slightly higher than the proportion of methylation QTLs in SLEs subtype–associated CpGs that did not change over time, but the difference was not statistically significant (n = 46 of 184 [25%]) (*P* = 0.179 by 2-sample test).

DISCUSSION

In this study, we examined the dynamics of DNA methylation in CpGs previously associated with SLE subtypes in a longitudinal cohort of SLE patients. Overall, we observed that a large proportion of SLE subtype–associated CpGs did not show significant change over 2 years. However, a much higher proportion of SLE subtype–associated CpGs changed over time compared to the genome-wide methylome. Some of the methylation changes observed over 2 years in SLE subtype–associated CpGs were associated with changes in cell-type proportions (26%) and medication use (4.5%).

Since the epigenome is not static, an important question related to EWAS is if associations may change over time. These results are encouraging, providing evidence that, overall, the methylation status of the majority of CpGs that were previously found to be associated with specific SLE subtypes remained unchanged over a 2-year period. Therefore, the blood methylome has potential as a biomarker for disease subtypes. This is further supported by findings from a recent longitudinal study examining DNA methylation in circulating granulocytes from SLE patients, where significant stability of the methylome was observed over a period of 4 years (38). With this in mind, we also refined our previous EWAS findings at the time of enrolment in the cohort by selecting CpGs that had the most robust difference in methylation between SLE subtypes and did not change over time. These candidate CpGs could be further prospectively studied at disease onset to determine their prognostic role in predicting SLE subtypes as well as their role as potential biomarkers for treatment response.

We observed a very small number of CpGs in which DNA methylation significantly changed over time. Interestingly, pathway analysis showed that most of these CpGs were involved in immune-related pathways such as intracellular viral sensing pathways, antigen processing, and IFN response as well as metabolic pathways (taurine metabolism, type II diabetes) (Table 3). We attempted to identify the underlying factors driving changes in DNA methylation. Overall, these changes were not correlated with disease activity, anti-dsDNA antibody titer, or lupus nephritis, although most individuals in our cohort had quiescent disease. Although most of the SLE subtype–associated CpGs were stable, there was a striking distinction between the increased proportion of SLE-associated CpGs that changed over time compared to genome-wide CpGs. One potential explanation for the progressive hypomethylation observed at SLE subtype–associated CpGs is that PBMCs in SLE patients have persistent exposure to cytokine milieu inherent in SLE, making immune-related CpGs in circulating PBMCs more susceptible to change. This is consistent with most EWAS that demonstrate hypomethylation of immune-related genes in SLE patients compared to healthy individuals, as well as severe SLE phenotypes compared to milder disease (5,8–10).

These findings have been attributed to defects in the enzymes responsible for the maintenance of DNA methylation (DNA methyltransferases) due to oxidative stress (39). Another potential hypothesis is that passive demethylation, the progressive loss of methylation over time, may be accelerated in SLE. The premise that passive demethylation can occur at different rates in individuals is the basis of epigenetic clocks or biomarkers of aging. These can drastically differ from chronological age (40). Whether accelerated passive demethylation, or epigenetic aging, occurs in SLE-relevant genes and pathways is unknown but should be examined in future longitudinal studies with longer periods of observation.

In studies with large sample sizes (>1,000), it is estimated that at least 10% and up to 45% of the methylome is influenced by nearby methylation QTLs (41). We investigated if genetic variation influenced methylation changes and found that a higher proportion of CpGs that changed over time were associated with methylation QTLs compared to stable CpGs. The leading hypothesis to explain cis-methylation QTL effects is that SNPs in protein binding sites alter or disrupt the activity of sequence-specific binding proteins such as transcription factors of methyl-binding

proteins which could lead to changes in methylation patterns of nearby CpGs (42–44). Since transcription factor binding is dynamic, one could hypothesize that this effect may influence the variability of methylation in addition to methylation itself. Other longitudinal methylation studies are needed to corroborate this observation.

As expected, when we examined our initial results in the unadjusted matrix for cell-type proportions, we found that a substantial proportion of changing CpGs (26%) were associated with at least 1 cell estimate. This is an important consideration for studies that use whole blood DNA methylation to study the epigenetic landscape in SLE. As cell-type proportions in peripheral blood samples are of biologic relevance to disease pathogenesis, we are faced with a conundrum: how to deal with a potential confounder that could itself be a disease outcome. In the current study, we addressed this by initially adjusting for cell-type proportions and then reexamining findings in an unadjusted matrix to assess the effects of cell-type proportions. Future studies of analytic approaches to whole blood DNA methylation data will be important given the low cost and feasibility of working with whole blood in comparison to sorted or single cells, particularly population-based studies that seek to provide a useful genomic clinical tool for precision medicine.

Limitations of this study include a relatively small sample size, which may have limited our ability to detect a larger number of CpG sites that varied over time or fully assess the association between self-reported ethnicity or race and genetic ancestry. In addition, the detection of methylation fluctuations associated with disease activity was not possible due to the fact that most participants had clinically inactive disease. Our study was underpowered to identify additional CpG sites associated with medication use. Finally, an extended interval of >2 years may have yielded different findings. However, there have been few studies that have reexamined cross-sectional DNA methylation associations in a longitudinal cohort. Our rigorous analysis pipeline addressed the potential limitations of studying whole blood DNA methylation in longitudinal studies, including effects of changes in cell-type proportions.

In summary, we characterized the DNA methylation dynamics of CpGs that were previously shown to be associated with SLE in this well-characterized CLUES longitudinal cohort. Among these SLE subtype–associated CpGs, we identified CpGs that remained stable over time. Given their association with SLE subtypes, these CpGs should be further evaluated to determine their potential role as biomarkers of disease outcomes. Additional longitudinal studies may also reveal whether SLE- and immune-related CpGs have accelerated passive demethylation in comparison to the genome-wide methylome. Future studies of methylome dynamics in SLE at the time of disease flare and remission may provide additional insight into epigenetic programs that may guide the development of precision medicine approaches for SLE.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lanata had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Lanata, Taylor, Chung, Sirota, Barcellos, Criswell.
Acquisition of data. Lanata, Solomon, Trupin, Katz, Dall'Era, Yazdany, Criswell.
Analysis and interpretation of data. Lanata, Nititham, Taylor, Chung, Blazer, Sirota, Barcellos, Criswell.

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