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Differential DNA methylation in Black and White individuals with chronic low back pain enrich different genomic pathways

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ABSTRACT

Compared to Non-Hispanic Whites (NHWs), individuals who self-identify as Non-Hispanic Blacks (NHBs) in the United States experience more severe and disabling chronic low back pain (cLBP). We hypothesized that differences in DNA methylation (DNAm) play a role in racial disparities in cLBP.

Purpose: To determine the relationship between DNAm levels and racial group differences in adults with cLBP. Our study's secondary purpose was to perform a race-stratified comparison of adults with cLBP and pain-free controls and identify functional genomic pathways enriched by annotated differentially methylated genes.

Patients and Methods: We recruited 49 NHBs and 49 NHWs (49 cLBP and 49 pain-free controls, PFCs), analyzed DNAm from whole blood using reduced representation bisulfite sequencing, and identified enriched genomic pathways.

Results: Among participants with cLBP, we identified 2873 differentially methylated loci (DML; methylation differences of at least 10% and $p < 0.0001$), many of which were annotated to genes of importance to pain pathology. These DMLs significantly enriched pathways to involved in nociception/pain processing (*Dopamine-DARPP32 Feedback in cAMP signaling*, *GABA Receptor Signaling*, *Opioid Signaling*) and neuronal differentiation (e.g., *Calcium Signaling, Axon Guidance Signaling*, and *Endocannabinoid Neuronal Synapse*). Our race stratified analyses of individuals with cLBP versus PFCs revealed 2356 DMLs in NHBs and 772 DMLs in NHWs with $p < 0.0001$ and $> 10\%$ methylation difference. Ingenuity Pathway Analysis revealed that many pathways of significance to pain such as *Corticotropin Releasing Hormone Signaling, White Adipose Tissue Browning,* and *GABA Receptor Signaling pathways*, were more significant in NHBs than NHWs.

Conclusion: Even though an individual's self-identified race is a social construct, not a biological variable, racism associated with that classification affects virtually every aspect of life, including disease risk. DNAm induced alterations in stress signaling pathways may explain worse pain outcomes in NHBs.

Introduction

Chronic low back pain (cLBP) is a significant public health problem in the United States and one of the leading causes of disability worldwide. (Clark and Horton, 2018; GBD, 2017) Despite its high prevalence, the disease process of cLBP is poorly understood. (Hartvigsen et al., 2018) An individual's race is one predictor of pain sensitivity, severity, and interference with daily activities. (Aroke et al., 2020a; Meints et al., 2018) Compared to non-Hispanic Whites (NHW), Non-Hispanic Blacks (NHB) experience more chronic pain, report more pain-related chronic medical conditions, more chronic pain-related disability, and have a more inferior pain-associated quality of life. (Kim et al., 2017; Edwards et al., 2001) The reasons for these racial differences in cLBP are poorly understood. This may be related to the fact that race is a socially

Abbreviations: NHB, Non-Hispanic Black; NHW, Non-Hispanic White; RRBS, Reduced representation bisulfite sequencing; cLBP, Chronic low back pain; PFC, Pain free control; DML, DIfferentially methylated loci; DNAm, DNA methylation.

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constructed categorization of people, different from biological traits such as ancestry, and not an inherent disease risk. (Aroke et al., 2019)

Genetic, (Battie et al., 1976; Battie et al., 2007) environmental, (Battie et al., 1976; Suri et al., 2017) and psychosocial factors (Moix et al., 2011; Pinheiro et al., 2016; Fernandez et al., 2017) have been implicated in the pathogenesis of cLBP. Studies have shown that psychosocial factors such as depressive symptoms, (Fernandez et al., 2017) catastrophizing, (Moix et al., 2011) and lower socioeconomic status (Hill and Fritz, 2011; Katz, 2006) predict worse cLBP outcomes. NHBs report more severe depressive symptoms and endorse more pain catastrophizing than NHWs. Also, NHBs are more likely to report lower socioeconomic status and lower social support than NHWs. NHBs experience more poverty, residential segregation, aggressive policing, interpersonal racial harassment, and microaggressions. (Sue et al., 2007; Williams, 2021) These psychosocial factors may contribute to racial differences in cLBP, but some studies have suggested that racial differences in pain persist after controlling for psychosocial factors. (Fuentes et al., 2007) NHBs are more likely to experience adverse environmental factors such as violence, racial discrimination, injustice, and adverse childhood experiences. (Nelson et al., 2018; Sack and Murphey, 2018; Clark et al., 1999) Differences in lived experience may thus contribute to racial differences in cLBP. Still, evidence of the mechanism by which psychosocial factors and adverse life exposures contribute to race differences in cLBP remains indeterminate.

Overwhelming evidence indicates that modifications in the human genome (epigenomic modifications) regulate what genes turn-on or turn-off leading to tissue differentiation. (Huang et al., 2014) Thus, epigenetic changes are critical in understanding the mechanisms of human physiologic and pathophysiologic states. (Kanherkar et al., 2014; Syed and Nemeroff, 2017) DNA methylation (DNAm) is a stable, heritable, and well-studied epigenetic modification. (Aroke et al., 2019; Dupont et al., 2009) Generally, DNAm is tissue-specific, however, in rat models with chronic pain, Massart *and colleagues* found a strong correlation between DNAm in the prefrontal cortex and peripheral blood T-cells; peripheral T-cell methylation predicted chronic pain with 80% accuracy. (Massart et al., 2016) Other researchers have also utilized peripheral blood methylation as a less-invasive sample to study methylation patterns in chronic pain conditions, including fibromyalgia, persistent post-mastectomy pain, generalized musculoskeletal pain, and cLBP. (Tajerian et al., 2011; Kawi et al., 2018; Sukenaga et al., 2016; Burri et al., 2016; Stephens et al., 2017)

We previously suggested that a lifetime of adversity, chronic stress, poor coping, and racial discrimination may induce epigenetic modifications, affecting adaptation and resulting in racial differences in adulthood disorders such as chronic pain. (Aroke et al., 2019) Emerging evidence suggests that DNAm changes that occur throughout the lifetime may play an essential role in the pathology of cLBP. (Gerra et al., 2017) *Tajerian and colleagues* have previously reported cLBP correlated with DNAm levels in secreted protein acidic and cysteine-rich (SPARC) gene. (Tajerian et al., 2011) Using reduced representation bisulfite sequencing (RRBS) and functional genomic enrichment analysis, we found several differentially methylated regions and important biologic pathways to be differentially enriched in individuals with cLBP compared to pain-free controls. The differentially methylated regions contained important genes such as *CELSR1, NAV1, MINK1,* and *KIF11*, which have previously been associated with pain, cell-cell adhesion/migration, and neural differentiation. (Aroke et al., 2020b) However, little is known about whether DNAm and functional genomic pathways vary as functions of an individual's self-identified race within the context of cLBP.

To gain insight into the role of epigenetic changes in racial differences in cLBP, we determined the relationship between DNAm levels and racial group differences in adults with cLBP. Specifically, we investigated how DNAm levels of NHBs with cLBP compare with those of NHWs with cLBP. Our study's secondary purpose was to perform a race-stratified comparison of adults with cLBP and pain-free controls. Furthermore, we performed functional genomic pathway analyses to identify biologically relevant pathways enriched by the differentially methylated genes.

Methods

Participants

Participants were enrolled as part of an ongoing study: Examining Racial And SocioEconomic Disparities in cLBP (R01MD010441). All participants were recruited through flyers posted at the Pain Treatment Clinic – University of Alabama at Birmingham (UAB) Department of Anesthesiology and the surrounding UAB community. Details about Enrollment criteria have been described in previous publications. (Aroke et al., 2020b; Penn et al., 2020) Briefly, we enrolled 25 NHWs and 25 NHBs with non-specific cLBP, ages of 19 and 85 years, from June 2018 to September 2019. To qualify as cLBP, participants must have experienced pain for at least half the days, for three or more consecutive months. Illnesses that could confound the interpretation of results were grounds for exclusion, including other pain conditions, malignancy, trauma, ankylosis for spondylitis, infection, poorly controlled diabetes, chronic inflammatory diseases (e.g., systemic lupus erythematosus, fibromyalgia, rheumatoid arthritis), severe psychiatric disorders requiring hospitalization in the last 12 months, and neurological disorders (e.g., epilepsy, multiple sclerosis, Parkinson's). The diagnosis was confirmed through medical records. The joint clinical practice guidelines for the American College of Physicians and the American Pain Society were used to identify non-specific cLBP in participants. (Chou et al., 2016)

For comparison, we also recruited and enrolled 50 (25 NHWs and 25 NHBs) pain-free controls (PFCs). Inclusion criteria for our pain-free controls included men and women 19 to 85 years of age, without a recent history of pain, not pregnant or breastfeeding, and able to write and read English. PFC participants were excluded using the same cLBP criteria. Participants with cLBP self-reported data about pain severity and pain interference were assessed using the Brief Pain Inventory (BPI) – Short Form, a widely used questionnaire to evaluate pain and its impact on functioning. (Cleeland and Ryan, 1994) The University of Alabama at Birmingham Institutional Review Board (IRB) for Human Subject Research approved this study. All participants had the opportunity to have any questions answered and provided written informed consent.

Measurement of DNA methylation

Peripheral whole blood samples were collected into ethylenediamine-tetra-acetic acid (EDTA) anticoagulant tubes. Genomic DNA was isolated using the *Gentra Puregene* DNA Purification Protocol (Qiagen, Valencia, CA, USA) and stored at −80 °C. The NanoDrop 2000 was used to quantify the extracted DNA. Determination of purity via spectrometry indicated an absorbance between 260 and 280 nm ratio of >1.8 for all samples.

The RRBS libraries were generated at the Heflin Center for Genomic Sciences at UAB, using the Ovation RRBS Methyl-Seq System (NuGEN, Tecan Genomics, Redwood City, CA, USA), according to the manufacturer's instructions. High-quality *MspI* digested genomic DNA fragments were ligated to adaptors and bisulfite converted (using the Qiagen Epitect kit for bisulfite conversion) strands sequenced on Illumina NextSq 500 platform to generate raw RRBS reads. The quantity and quality of the libraries were assessed using a Qubit fluorometer.

Data processing and analysis

Demographic and clinical data were summarized and compared using means (with standard deviation) and frequency (with percentage) depending on the level of measurement. Quality control (QC) of read was performed using FastQC. (Fast, 2019) We used Trim Galore (Trim,

2019) to trim low quality reads, and remove adapters before aligning and mapping to the human reference genome (hg19) using Bismark. (Krueger and Andrews, 2011) DNA methylation level of each locus was further extracted based on the aligned reads for the downstream statistical analysis.

To test the association of DNAm with cLBP status within each racial group, we performed differential methylation analyses comparing cLBP versus PFC within NHBs group and NHWs, respectively. Specifically, we employed linear regression models followed by the empirical Bayes moderated *t*-statistics test, which were implemented in the R *limma* package. (Ritchie et al., 2015) In this model, the methylation level of a CpG loci was the outcome variable, the pain status (cLBP or PFC) was the predictor variable, adjusting for age and sex as covariates. Differentially methylated loci (DML) were defined as methylation differences of at least 10% and $p < 0.0001$. Hypomethylated DMLs were those with significantly lower percent methylation in cLBP than in PFCs; and hypermethylated DMLs with significantly higher percent methylation in cLBP than in PFCs. To correct for multiple testing, we used the Benjamini-Hochberg method to determine q-values, (Wang et al., 2011) which is the equivalence of the false discovery rate (FDR). (Akalin et al., 2012) We annotated the location of DMLs in relation to genomic features (intron, exons, intergenic regions, and promoters regions) or CpG features (CpG island, CpG shores). CpG island features included CG fractions > 0.5, CG length of at least 200 bp, and an observed to expected CpG ratio of > 0.6. CpG shores were identified as positions adjacent to CpG islands with a length of at least 2000 bp. All these annotation analyses were performed using R *methylKit* package. (Akalin et al., 2012)

To examine the race disparity in relation to methylation level of subjects with cLBP, we performed differential methylation analysis comparing NHWs and NHBs within cLBP participants similarly using linear regression via R *limma* package. To be specific, the race (NHW or NHB) was the predictor variable, the methylation level of a CpG site was the outcome, adjusting for age and sex as covariates. To correct for multiple testing, we used the Benjamini-Hochberg method to obtain q-values. Hypomethylated DMLs were defined as those with significantly lower percent methylation NHBs and hypermethylated DMLs with significantly higher percent methylation in NHBs. The putative DMLs were annotated to genomic features (intron, exons, intergenic regions and promoter regions) or CpG features (CpG island, CpG shores).

To examine whether the association between pain and methylation were moderated by race, we further evaluated the pain-race interaction. We adopted linear regression models via R *limma* package. In this model, a methylation level for each CpG site was the outcome variable, race, pain status, and pain by race interaction were predictors, and age and gender were covariates. A significant pain by race interaction would imply the pain effect on methylation level could be different by race. As no DMLs from the interaction analysis were found to be statistically significant after correcting for multiple testing, we defined DMLs as nominal $p < 0.0001$.

To identify potential pathways driving racial disparities in pain, we used functional enrichment analyses to determine if any biological processes were overrepresented in genes based on DMLs. Following the removal of duplicates, genes annotated to DMLs showing significant differences ($p < 0.0001$) were imported into Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA), and genomic pathways over-represented by these genes determined. The analyses were carried out for (i) race stratified analysis in the association of DNAm with cLBP status; (ii) race disparity analysis in relation to methylation level of subjects with cLBP; and (iii) pain-race interaction analysis. Over-represented pathways in IPA with p-values < 0.01 ($-\log_{10}$p-value = 2) were considered statistically significant.

Results

Characteristics of study participants

Of the 100 participants recruited, two participants were excluded from analysis because they selected multiple racial backgrounds. Our final sample included 49 NHBs and 49 NHWs. The participants' mean age was 44.6 ± 12.8 years; there were slightly more women (53.1%) than men (46.9%). There was no statistically significant racial group difference in terms of age or gender of participants. Table 1 describes demographics and a description of pain outcomes. On average, NHBs reported greater pain severity and interference compared to NHWs with cLBP. The difference in pain severity was statistically significant ($p < 0.001$).

Quality of the samples and reproducibility of the data

Each of the 103 data files comprised, on average, approximately 45 million trimmed sequence reads, with each file about 7 GB in size. Among these 103 samples, there were 5 technical replicates and 98 unique biological samples. Each file was mapped to the reference human genome (hg19) using bismark to build bisulfite genome libraries. The bisulfite conversion rate was >99% for all study samples. The mapping efficiency ranged from 68% to 75%, consistent with other research using bismark to map bisulfite sequence data. DNAm profiles of the five randomly selected biological replicates matched perfectly. As depicted in Supplemental Fig. 1, a pairwise comparison of the technical samples revealed a correlation coefficient of>0.9 for all comparisons, which indicates high levels of reproducibility. (Bock, 2012)

Race stratified DNA methylation of adults with and without cLBP

We have previously identified DMLs and differentially enriched biological pathways in a mixed racial group of adults with cLBP versus PFCs. (Aroke et al., 2020b) To further explicate the role of DNAm in racial differences in cLBP, we compared DMLs and over-represented pathways within each racial group: NHBs (cLBP versus PFC) and NHWs (cLBP and PFC). Fig. 1 depicts an overview of the genome-wide

Table 1
Characteristics of study participants.

	Non-Hispanic Blacks	Non-Hispanic Whites	p-values
Chronic Low Back Pain Cases			
	(n = 25)	(n = 24)	
Age, mean (SD)	43.5 (10.6)	45.8 (14.9)	0.541
Sex, N (%)			
Men	9 (36)	12 (50)	0.321
Women	16 (64)	12 (50)	
BPI, mean (SD)			
Severity	5.9 (2.2)	3.4 (1.9)	<0.001
Interference	4.4 (2.8)	2.8 (2.0)	0.077
Pain-Free Controls			
	(n = 24)	(n = 25)	
Age, mean (SD)	40.7 (16.5)	39.3 (12.6)	0.749
Sex, N (%)			
Men	13 (54.2)	12 (48)	0.778
Women	11 (45.8)	13 (52)	
BPI, mean (SD)			
Severity	0.1 (0.5)	0.1 (0.1)	0.891
Interference	0.1 (0.5)	0	0.19

Notes: SD = standard deviation, BPI = brief pain inventory.

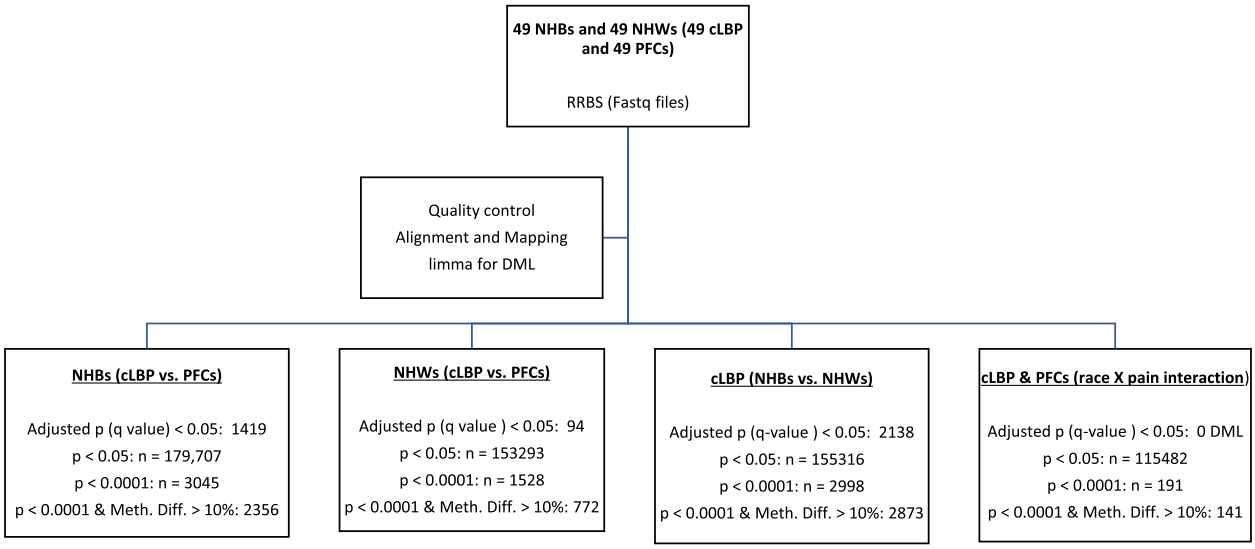

Fig. 1. Overview of the genome wide differential methylation analysis. Notes: NHBs = Non-Hispanic Blacks; NHWs = Non-Hispanic Whites; cLBP = chronic low back pain; PFCs = pain-free controls; Meth.Diff = absolute methylation difference between two groups; DML = differentially methylated loci.

differential methylation analysis.

Differentially methylated loci between cLBP- and PFCs in NHBs

After controlling for age and sex, differential methylation analysis revealed 3045 DMLs exhibiting significant differences at $p < 0.0001$, and 1419 at $q < 0.05$ in cLBP vs. PFCs NHBs. Of the 3045 DMLs, 2851 were hypomethylated and 194 were hypermethylated. Also, 2356 DMLs had an absolute methylation difference of >10 percent and $p < 0.0001$ in cLBP vs. PFC NHBs. Table 2 summarizes the top 20 DMLs that significantly predict cLBP in NHBs sorted by the p-values. Detailed examination of the DMLs revealed that majority mapped to intronic region (33%), followed by intergenic region (31%), promoter regions (25%), and exonic regions (10%) (Supplemental Fig. 2A). Also, 29% of the DMLs belonged to CpG island, 18% of the DMLs belonged to CpG shores (Supplemental Fig. 2B).

Pathway analysis of DMLs in NHBs

To infer the functional role of the identified DMLs, we performed a pathway enrichment analysis of genes annotated to DMLs with p-values <0.0001. From the list of 2110 differentially methylated genes, IPA mapped 110 canonical pathways ($p < 0.01$) over-represented in the NHB (cLBP versus PFC) data set. Many of the overrepresented pathways are of relevance to pain (e.g., opioid signaling), chronic stress (e.g., corticotropin releasing hormone signaling, white adipose tissue browning, and dopamine-DARPP32 Feedback in cAMP signaling) and neuronal differentiation (e.g., synaptogenesis signaling, calcium signaling, and CREB signaling in neurons). Fig. 2 depicts the top 20 over-represented canonical pathways from differentially methylated genes between NHBs with CLBP and NHB PFCs. Supplemental Table 1 summarizes the differentially methylated genes associated with the over-represented pathways.

Table 2
Top 20 Differentially Methylated Loci Between cLBP Vs. PFC in NHBs.

Chr	Position	Beta	SE	95% CI		P-value	q-value	Genes	Gene name/description	Genomic Features
				LL	UL					
3	186,629,673	−0.19	0.02	−0.23	−0.14	6.39E-11	1.66E-04			intergenic
2	121,106,655	−0.21	0.03	−0.26	−0.16	1.96E-10	2.48E-04	INHBB	Inhibin Subunit Beta B	intergenic
6	111,591,940	−0.16	0.02	−0.21	−0.12	2.86E-10	2.48E-04	KIAA1919	Sodium-Dependent Glucose Transporter	exons
15	102,255,559	−0.11	0.01	−0.14	−0.08	3.94E-10	2.56E-04	TARSL2	Threonyl-tRNA Synthetase-Like 2	intergenic
12	20,922,311	0.18	0.02	0.13	0.22	7.14E-10	3.71E-04			intergenic
10	9,595,069	−0.19	0.02	−0.24	−0.14	8.59E-10	3.72E-04			intergenic
8	144,408,266	0.35	0.05	0.26	0.44	1.24E-09	4.14E-04	TOP1MT	DNA Topoisomerase I Mitochondrial	introns
2	242,428,016	−0.16	0.02	−0.20	−0.12	1.28E-09	4.14E-04	FARP2; STK25	Serine/Threonine Kinase 25	introns
21	10,597,920	−0.16	0.02	−0.21	−0.12	2.37E-09	6.84E-04			intergenic
1	10,670,667	−0.10	0.01	−0.13	−0.07	2.92E-09	7.59E-04	PEX14	Peroxisomal Biogenesis Factor 14	introns
19	40,177,900	−0.17	0.02	−0.22	−0.12	6.87E-09	1.62E-03	LGALS17A	Galectin 14 pseudogene	intergenic
11	8,285,031	−0.24	0.03	−0.31	−0.17	9.18E-09	1.89E-03	LMO1	LIM Domain only 1; Rhombotin 1	intergenic
9	100,683,907	−0.27	0.04	−0.35	−0.19	9.46E-09	1.89E-03	C9orf156	Chromosome 9 open reading frame 156	intergenic
10	35,465,538	−0.10	0.02	−0.14	−0.07	1.08E-08	2.01E-03	CREM	cAMP Responsive Element Modulator	introns
14	24,951,201	0.35	0.05	0.25	0.46	1.70E-08	2.82E-03			intergenic
12	28,343,050	−0.31	0.05	−0.41	−0.22	1.84E-08	2.82E-03	CCDC91	Coiled-Coil Domain Containing 91	promoters
17	8,926,988	−0.26	0.04	−0.33	−0.18	1.85E-08	2.82E-03	NTN1	Netrin 1	introns
5	176,166,533	−0.25	0.04	−0.32	−0.17	2.24E-08	2.99E-03	RP11-375B1.2	Long noncoding RNA	intergenic
15	86,118,741	−0.17	0.02	−0.22	−0.12	2.40E-08	2.99E-03	AKAP13	A-Kinase Anchor Protein 13	intergenic
19	10,297,587	0.25	0.04	0.17	0.32	2.44E-08	2.99E-03	DNMT1	DNA Methyltransferase 1	introns

Notes: Chr = chromosome, CI = confidence interval; LL = lower limit of the confidence interval; UL = upper limit of the confidence interval; SE = standard error; cLBP = chronic low back pain; PFC = pain-free control; NHBs = non-Hispanic Blacks.

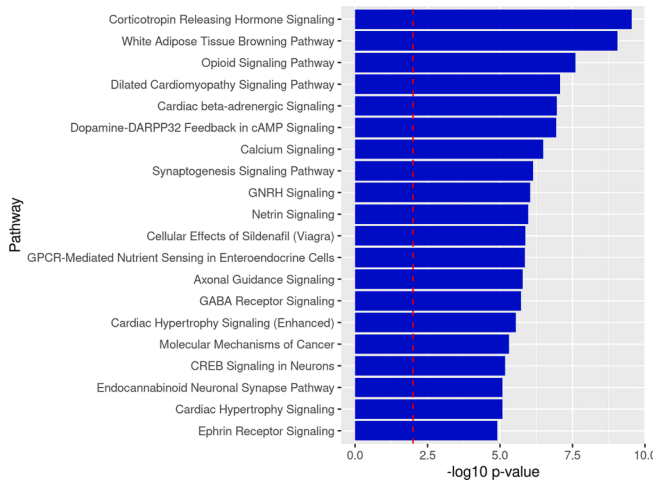

Fig. 2. Top 20 Over-represented pathways by identified from genes containing DMLs between cLBP and PFCs in NHBs. Note: The dotted line depicts the statistically significant level ($p < 0.01$).

Differentially methylated loci between cLBP- and PFCs in NHWs

After controlling for age and sex, we identified 1528 (1451hypo-methylatedand77hypermethylated) DMLs with p values < 0.0001, and 94 DMLs with q values < 0.05 in models predicting cLBP in NHWs. Of the 1528 DMLs, 772 had a methylation difference of at least 10 percent in NHWs with cLBP compared to NHW PFCs. Table 3 summarizes the annotated genes of the top 20 DMLs based on smallest p-values. Detailed examination of the DMLs revealed that majority mapped to intronic (32%), followed by intergenic regions (30%), promoter regions (28%), and exon regions (10%) (Supplemental Fig. 3A). In addition, 33% of the DMLs belonged to CpG island, 17% of the DMLs belonged to CpG shores (Supplemental Fig. 3B).

Pathway analysis of DMLs in NHWs

From the list of 1330 differentially methylated genes annotated to DMLs with $p < 0.0001$, IPA mapped over-represented pathways. IPA revealed 31 canonical pathways over-represented ($p < 0.01$) from the list of differentially methylated genes. Top over-represented pathways were related to inflammation and immunity (notch signaling, role of JAK family kinases in IL-6-type Cytokine signaling and IL-22 signaling), and neuronal differentiation (synaptogenesis signaling and axon guidance signaling) pathways (Fig. 3). Supplemental Table 2 summarizes the

Table 3
Top 20 Differentially Methylated Loci Between cLBP vs. PFCs in NHWs.

Chr	Position	Beta	SE	95% CI		P-value	q-value	Genes	Gene Name/Description	Genomic Features
				LL	UL					
15	102,255,559	−0.13	0.02	−0.17	−0.10	2.24E-10	5.81E-04	TARSL2	Threonyl-tRNA Synthetase-Like 2	intergenic
1	148,556,709	−0.10	0.01	−0.13	−0.07	5.02E-09	6.52E-03	NBPF15	Neuroblastoma breakpoint family, member 15	intergenic
17	47,633,779	−0.14	0.02	−0.18	−0.10	3.06E-08	1.87E-02	RP5-1029 *K*10.2	Long non-coding RNA	intergenic
12	40,499,027	−0.08	0.01	−0.11	−0.06	3.48E-08	1.87E-02	SLC2A13	Solute Carrier Family 2 Member 13	promoters
21	46,975,705	−0.10	0.02	−0.13	−0.07	3.83E-08	1.87E-02	–		introns
11	2,009,641	−0.22	0.03	−0.29	−0.15	4.31E-08	1.87E-02	MRPL23	Mitochondrial Ribosomal Protein L23	introns
10	119,304,081	−0.24	0.04	−0.32	−0.16	6.71E-08	2.25E-02	EMX2	Empty Spiracles Homeobox 2	promoters
17	40,718,967	−0.21	0.03	−0.27	−0.14	7.70E-08	2.25E-02	COASY; MLX	Coenzyme A Synthase; Max-Like Protein X	promoters
20	8,942,669	−0.07	0.01	−0.10	−0.05	7.81E-08	2.25E-02	PLCB1	Phospholipase C Beta 1	intergenic
19	36,736,335	−0.09	0.01	−0.12	−0.06	1.02E-07	2.66E-02	ZNF565	Zinc Finger Protein 565	intergenic
16	1,382,020	−0.14	0.02	−0.18	−0.09	1.19E-07	2.79E-02	UBE2I; BAIAP3	Ubiquitin Conjugating Enzyme E2 I	intergenic
1	222,638,844	−0.06	0.01	−0.09	−0.04	1.29E-07	2.79E-02	CICP13	capicua transcriptional repressor pseudogene	intergenic
1	167,424,858	−0.15	0.02	−0.20	−0.10	1.53E-07	2.85E-02	CD247	T-cell receptor coding	intergenic
2	44,223,044	−0.07	0.01	−0.10	−0.05	1.64E-07	2.85E-02	LRPPRC	Leucine Rich Pentatricopeptide Repeat Containing Protein	intergenic
2	111,877,924	−0.10	0.02	−0.13	−0.07	1.67E-07	2.85E-02	ACOXL; BCL2L11	Acyl-CoA Oxidase Like	intergenic
18	40,656,876	−0.14	0.02	−0.19	−0.09	1.97E-07	2.85E-02	RIT2	Ras Like Without CAAX 2	introns
16	88,497,586	−0.21	0.03	−0.27	−0.14	2.03E-07	2.85E-02	ZNF469	Zing-finger protein 469	intergenic
15	96,885,264	−0.11	0.02	−0.15	−0.07	2.10E-07	2.85E-02	NR2F2	Nuclear Receptor Subfamily 2 Group F Member 2	intergenic
13	107,186,569	−0.18	0.03	−0.24	−0.12	2.30E-07	2.85E-02	EFNB2	Ephrin B2	intergenic
18	19,746,392	−0.13	0.02	−0.18	−0.09	2.32E-07	2.85E-02	GATA6-AS1; GATA6	GATA Binding Protein 6	intergenic

Notes: Chr = chromosome, CI = confidence interval; LL = lower limit of the confidence interval; UL = upper limit of the confidence interval; SE = standard error; cLBP = chronic low back pain; NHW = non-Hispanic White.

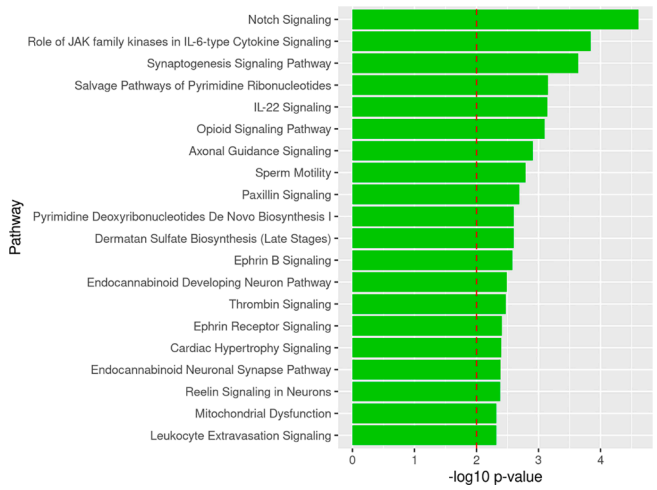

Fig. 3. Top 20 Over-represented pathways by identified from genes containing DMLs between cLBP and PFCs in NHWs. Note: The dotted line depicts the statistically significant level ($p < 0.01$).

list of over-represented pathways from differentially methylated genes between NHWs with cLBP and PFCs.

Comparing over-represented pathways in race stratified analysis

As previously mentioned, IPA revealed 110 and 31 overrepresented pathways among genes containing significant DMLs between cLBP cases and PFCs in NHBs and NHWs, respectively. For comparison, we classified the top 20 over-represented pathways according to calculated p-values. As depicted in Fig. 4, most of the calculated p-values were more significant in NHBs than NHW data set, suggesting that these pathways have a larger effect size in NHBs than NHWs.

Differentially methylated loci in associated with cLBP in NHBs versus NHWs

To investigate differential methylation between NHBs and NHWs related to cLBP, we used linear models to predict DML using race,

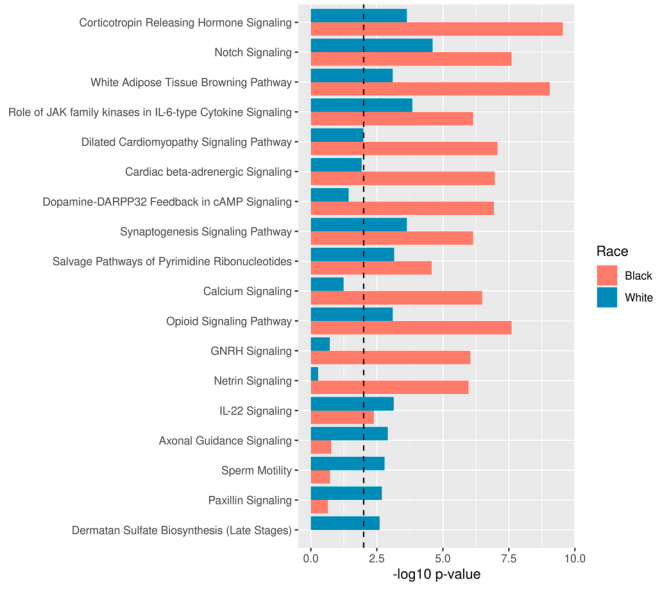

Fig. 4. Comparison of top the 20 over-represented pathways in race stratified analysis. Note: The dotted line depicts the statistically significant level ($p < 0.01$).

controlling for sex, and age. At $p < 0.0001$ level, we identified 2998 (1458hypomethylatedand1540hypermethylated) DMLs. Of these, 2873 DMLs had at least a 10 percent methylation difference between NHBs and NHWs with cLBP. After controlling for multiple testing, 2138 DMLs remained statistically significant ($q < 0.05$). The top 20 DMLs identified according to the order of p-values from low to high are shown in Table 4. The data indicated that majority of the mapped to intronic region (38%), followed by intergenic regions (34%), promoter regions (18%) and exon regions (10%) (Supplemental Fig. 4A). 61% of the DMLs belonged to CpG island, 22% of the DMLs belonged to CpG shores (Supplemental Fig. 4B).

Pathway analysis of DMLs between NHBs and NHWs with cLBP

To investigate the potential physiological processes that influence racial differences in epigenetic changes associated with cLBP, 1695 genes containing significant DMLs with $p < 0.0001$ were further analyzed using the IPA software. At p-value < 0.01, IPA revealed 79 overrepresented pathways from the list of differentially methylated genes. Many of the top overrepresented pathways are of relevance to nociception/pain processing (corticotropin releasing hormone signaling, dopamine-DARPP32 feedback in cAMP signaling, GABA receptor signaling, opioid signaling) and neuronal differentiation (e.g., synaptogenesis signaling pathway, calcium signaling, axon guidance signaling, and endocannabinoid neuronal synapse). Fig. 5 shows the list of top 20 over-represented pathways from genes containing DMLs between NHB and NHW individuals with cLBP. The differentially methylated genes overrepresented in the pathways are shown in Supplemental Table 3.

Differentially methylated loci by race-pain interaction

Analysis of the race by pain interaction effects identified 191 statistically significant DMLs at $p < 0.0001$. These DMLs annotated to important regulatory genes and those of relevance to pain pathology, including *ELL2, CTD-3179p9.2, ZNF808, SYN3, SOX7*, and *LRFN5*. Table 5 summarizes the top 20 DMLs by race-pain interaction. However, at 5% false discovery rate (FDR) there is no DML, implying no interaction effect between race and pain. Future large studies are needed to replicate these results.

Pathways analysis of genes containing DMLs in race-pain interactions

After removing duplicate, 160 genes containing DMLs with $p < 0.0001$ were subjected to IPA to determine functional canonical pathways enriched by the interactions of race, pain and DML in NHBs and NHW with cLBP. We identified 34 pathways that were significantly enriched by the genes ($p < 0.01$). Fig. 6 depicts the top 20 enriched pathways, including those relevant to pain pathologies such as axon guidance signaling, endocannabinoids neuronal synapse, calcium signaling, and Wnt/beta-catenin signaling pathways. Supplemental Table 4 summarizes the list of over-represented pathways and associated differentially methylated genes containing DMLs with race-by-pain interactions.

Discussion

Epigenetic modifications, particularly DNAm, induced by environmental exposures may be responsible for heritable changes in gene expression and account for a substantial fraction of variability in physiologic and disease processes. (van Dongen et al., 2016) Multiple studies have documented an elevated risk for many chronic diseases, including cLBP among NHBs. (Aroke et al., 2020a; Meints et al., 2018) While an individual's race is not a biological trait or an inherent disease risk, societal racism, environmental exposures, and chronic stress may contribute to racial disparities in pain through epigenetic markings on

Table 4
Top 20 Differentially Methylated Loci in Associated with cLBP in NHBs versus NHWs.

Chr	Position	Beta	SE	95% CI		P-value	q-value	Genes	Gene Name/Description	Genomic Features
				LL	UL					
1	1,419,278	0.76	0.05	0.66	0.87	1.20E-18	3.12E-12	ATAD3B	ATPase Family AAA Domain Containing 3B	intergenic
17	36,590,735	0.71	0.05	0.60	0.81	8.82E-18	1.14E-11	ARHGAP23	Rho GTPase Activating Protein 23	intergenic
18	11,550,683	0.69	0.06	0.58	0.81	3.69E-16	3.19E-10	RP11-712C7.2	Long-noncoding RNA	intergenic
5	176,190,238	−0.67	0.06	−0.78	−0.56	7.66E-16	4.97E-10			intergenic
1	53,970,911	0.72	0.06	0.60	0.85	1.68E-15	8.74E-10	GLIS1	GLIS Family Zinc Finger 1	intergenic
19	4,028,691	0.70	0.06	0.57	0.83	1.93E-14	8.37E-09	PIAS4	Protein Inhibitor Of Activated STAT 4	intergenic
6	95,534,202	0.73	0.07	0.59	0.86	3.08E-14	1.14E-08			intergenic
1	160,347,421	0.68	0.06	0.55	0.80	4.60E-14	1.39E-08	NHLH1	Nescient Helix-Loop-Helix 1	intergenic
12	50,862,234	−0.73	0.07	−0.87	−0.59	4.80E-14	1.39E-08	LARP4	La Ribonucleoprotein 4	intergenic
20	62,149,177	0.64	0.06	0.52	0.76	6.01E-14	1.56E-08	PPDPF	Pancreatic Progenitor Cell Differentiation And Proliferation Factor	intergenic
18	71,841,430	−0.61	0.06	−0.73	−0.49	1.47E-13	3.48E-08			intergenic
10	127,291,526	0.61	0.06	0.49	0.73	2.82E-13	6.10E-08	TEX36	Testis Expressed 36	introns
4	177,637,599	−0.54	0.06	−0.66	−0.43	6.64E-13	1.33E-07	VEGFC	Vascular Endothelial Growth Factor C	intergenic
10	128,944,081	−0.64	0.07	−0.77	−0.51	7.26E-13	1.35E-07	DOCK1	Dedicator Of Cytokinesis 1	introns
15	74,592,862	−0.60	0.06	−0.73	−0.48	1.10E-12	1.82E-07	CCDC33	Coiled-Coil Domain Containing 33	intergenic
2	195,597,960	0.38	0.04	0.30	0.46	1.12E-12	1.82E-07	AC006196.1	Long non-coding RNA	intergenic
19	51,801,429	0.67	0.07	0.53	0.81	1.51E-12	2.30E-07			intergenic
4	3,578,876	0.36	0.04	0.28	0.44	3.67E-12	5.28E-07	LINC00955	Long non-coding RNA	promoters
1	11,395,415	0.67	0.07	0.52	0.81	3.86E-12	5.28E-07			intergenic
1	16,455,052	−0.64	0.07	−0.78	−0.50	5.51E-12	7.16E-07	EPHA2	Ephrin type A receptor 2	intergenic

Notes: Chr = chromosome, CI = confidence interval; LL = lower limit of the confidence interval; UL = upper limit of the confidence interval; SE = standard error; cLBP = chronic low back pain; NHW = non-Hispanic White; NHBs = non-Hispanic Blacks.

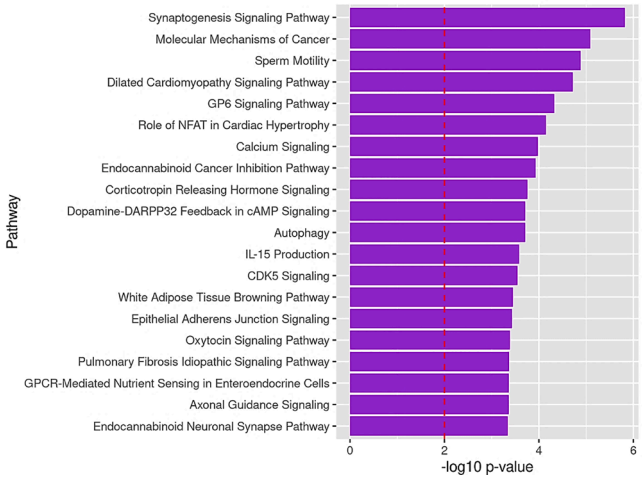

Fig. 5. Top 20 over-represented pathways by genes containing DMLs between NHBs and NHWs with cLBP. Note: The dotted line depicts the statistically significant level ($p < 0.01$).

the DNA, thus linking lived experience and cLBP risk. (Aroke et al., 2019) Other investigators have supported the potential mechanistic role of epigenetic modifications as "biological memories of experiences acquired earlier in our own lives" and environment (p. 801). (Thayer and Kuzawa, 2011) Specifically, epigenetic changes can link environmental exposures to biological processes that affect disease risk. Using RRBS analysis followed by pathway analysis, we identified DMLs between NHBs and NHWs with cLBP and over-represented pathways from genes containing the DMLs, thus revealing potential molecular pathways implicated in racial disparities in cLBP.

To our knowledge, this is the first study to apply RRBS to investigate associations between DNAm and racial disparities in cLBP and use integrative computational analysis to identify functional pathways over-represented by the genes with DMLs. Our results demonstrate well-defined racial category-associated differential methylation patterns among individuals with cLBP. As expected, our genome-wide methylation analysis revealed DML in various regions, including intronic, intergenic, promoters, and exons, as well as CpG island and shores. Generally, methylation of CpGs in the promoter region of genes blocks transcription factors from binding and inhibits gene expressions. Typically CpG islands span 200–1000 base pairs and can overlap the first intron with gene promoter regions, regulating transcription. (Moore et al., 2013) However, the effect of methylation in intergenic or non-promoter gene regions on gene expression is less straightforward.

Table 5
Top 20 Differentially Methylated Loci Based on Race-Pain Interaction Effects.

Chr	Position	Beta	SE	95% CI		P-values	q-value	Genes	Gene Name/Description	Genomic Features
				LL	UL					
12	103,311,112	−0.09	0.02	−0.12	−0.05	1.84E-06	0.96	PAH	Phenylalanine Hydroxylase	promoters
5	95,296,026	0.22	0.04	0.13	0.3	2.14E-06	0.96	ELL2	Elongation Factor For RNA Polymerase II 2	introns
20	61,185,059	0.29	0.06	0.17	0.41	4.36E-06	0.96	–		intergenic
5	117,618,581	−0.16	0.03	−0.22	−0.09	5.76E-06	0.96	CTD-3179P9.2	Long Intergenic Non-Protein Coding RNA	intergenic
19	53,031,512	−0.16	0.03	−0.23	−0.1	5.93E-06	0.96	ZNF808	Zinc Finger Protein 808	intergenic
1	195,667,563	0.15	0.03	0.09	0.21	5.94E-06	0.96	–		intergenic
16	31,342,748	−0.22	0.05	−0.32	−0.13	6.24E-06	0.96	ITGAM	Integrin Subunit Alpha M	introns
3	53,301,594	−0.06	0.01	−0.09	−0.04	8.13E-06	0.96	–		intergenic
12	8,046,968	0.11	0.02	0.06	0.16	8.98E-06	0.96	SLC2A14	Solute Carrier Family 2 Member 14	intergenic
22	32,955,596	−0.15	0.03	−0.21	−0.08	9.20E-06	0.96	SYN3	Synapsin III	intergenic
1	228,318,664	0.11	0.02	0.06	0.16	1.00E-05	0.96	RP11-520H14.1	Pseudogene	intergenic
11	117,665,622	−0.33	0.07	−0.47	−0.19	1.01E-05	0.96	DSCAML1	Down Syndrome Cell Adhesion Molecule Like 1	intergenic
8	10,589,079	−0.12	0.02	−0.17	−0.07	1.01E-05	0.96	SOX7	SRY-related HMG-Box Transcription Factor 7	introns
17	3,790,089	0.3	0.07	0.17	0.44	1.08E-05	0.96	CAMKK1	Calcium/Calmodulin Dependent Protein Kinase Kinase 1	intergenic
1	205,023,204	0.23	0.05	0.13	0.33	1.16E-05	0.96	CNTN2	Contactin 2	introns
14	42,074,670	0.06	0.01	0.04	0.09	1.18E-05	0.96	LRFN5	Leucine Rich Repeat and Fibronectin Type III domain containing 5	intergenic
7	1,867,522	−0.09	0.02	−0.13	−0.05	1.32E-05	0.96	MAD1L1	Mitotic Arrest Deficient 1 like 1	intergenic
3	179,390,332	−0.05	0.01	−0.08	−0.03	1.37E-05	0.96	USP13	Ubiquitin Specific Peptidase 13	intergenic
1	2,392,971	0.16	0.03	0.09	0.23	1.39E-05	0.96	PLCH2	Phospolipase C eta 2	intergenic
20	44,552,516	−0.05	0.01	−0.08	−0.03	1.47E-05	0.96	–		intergenic

Notes: Chr = chromosome, CI = confidence interval; LL = lower limit of the confidence interval; UL = upper limit of the confidence interval; SE = standard error; cLBP = chronic low back pain.

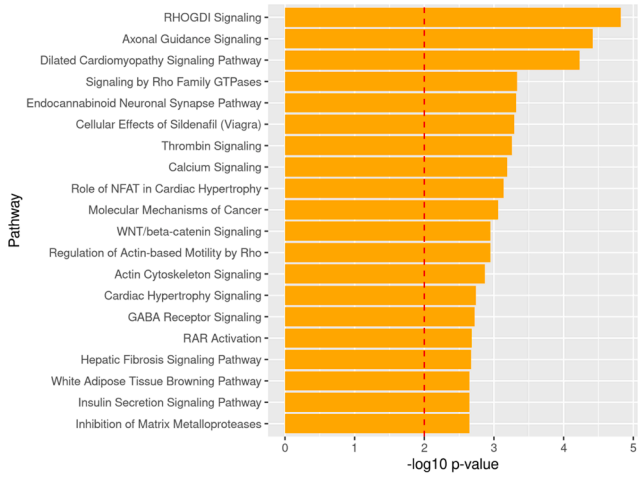

Fig. 6. Top 20 over-represented pathways by genes containing DMLs from the race by pain interaction among adults with cLBP. Note: The dotted line depicts the statistically significant level ($p < 0.01$).

Thus, given the high number of annotated differentially methylated genes and varied distribution of the DMLs, we will focus our discussion on the over-represented pathways.

Our findings suggest that differentially methylated genes between NHBs and NHWs with cLBP were over-represented in pathways involved in chronic pain, chronic stress, and the brain reward system, notably, *Corticotropin-Releasing Hormone Signaling, Dopamine-DARPP32 Feedback in cAMP Signaling,* and *White Adipose Tissue Browning Pathway*. At the physiological level, chronic stress and chronic pain share several overlapping processes. (Abdallah and Geha, 2017) Enrichment of *Corticotropin-Releasing Hormone Signaling* and *Dopamine-DARPP32 Signaling* pathways have previously been linked with chronic postsurgical pain in adolescents undergoing spinal surgery. (Chidambaran et al., 2019) Others have reported that long-term exposure to stress and early-life adversity correlate with epigenetically induced alterations in related stress adaptation pathways. (Silberman et al., 2016; Géranton, 2019) Also, chronic pain and stress can induce depressive symptoms, potentially through epigenetically induced long-term neuroplasticity in the central nervous system. For instance, epigenetic alterations in pathways involved in dopamine metabolism have been associated with depressive symptoms and chronic pain conditions. (Géranton, 2019; Kerr and Burri, 2017) Similarly, Sidossis and colleagues reported that prolonged stress epigenetically induces browning of subcutaneous white adipose tissue in children recovering from burns. (Sidossis et al., 2015) Consistent with

our previous hypothesis, (Aroke et al., 2019) these findings suggest the potential role of chronic stress, such as the stress of racial discrimination on racial disparities in cLBP.

Our sample observed that *Synaptogenesis Signaling, Wnt/β-catenin Signaling, Axon Guidance Signaling, Netrin Signaling*, and *Calcium Signaling* pathways were over-represented in genes differentially methylated between NHBs and NHWs with cLBP. This observation aligns with previous publications indicating that disruptions in neuronal proliferation and sensitizing could play a role in chronic pain. (Babcock et al., 2011; Yang et al., 2020; Tang, 2014; Zhao and Yang, 2018; Patil and Andry, 2017; Shi et al., 2016) Netrin and Wnt signaling play an essential role in neuronal development (axon guidance) through a multifunctional protein complex that interacts with transcriptional factors and affects target gene expression. (Nusse, 2005; Gao et al., 2020) Wnt signaling ligands and receptors (e.g., Frizzled family of receptors) are expressed along neural pain pathways in the dorsal root ganglion (DRG) (Tang, 2014) and play a role in the etiology of neuropathic and inflammatory pain. (Zhang et al., 2013; Wu et al., 2020) Using animal models, Zheng and colleagues reported that nerve injury and bone cancer increase Wnt expression and β-catenin levels in the sensory neurons in the DRG. At the same time, spinal blockade of Wnt signaling suppressed the induction of pain and delayed pain processing for up to 14 days. (Zhang et al., 2013) Other investigators have also found that increased expression of *WNT5B* plays an essential role in the transition from acute to chronic pain and the development of opioid withdrawal symptoms. (Wu et al., 2020) Similarly, increased expression of Netrin signaling pathways appears to be neuroprotective after spinal code injury. (Gao et al., 2020) Chronic stress affects genes' expression in Netrin and Wnt signaling pathways and has been associated with anxiety-like behavior, attention deficit hyperactivity disorder, insulin resistance, depressive symptoms, and increased blood pressure. (Odaka et al., 2017; Yde Ohki et al., 2020; Torres-Berrío et al., 2020) Thus, it is plausible that higher levels of perceived stress (e.g., the stress of racial discrimination) induce alterations in Netrin and Wnt signaling pathways and result in higher cLBP outcomes in NHBs.

*Glu*tamate, γ-amino-butyric acid (*GABA) Receptor Signaling, Opioid Signaling, Endocannabinoid Neuronal Synapse,* and *G-Protein Couple Receptor-Mediated Nutrient Sensing in Enteroendocrine cells* pathways were over-represented. It is well known that neurotransmitters such as GABA, substance P, endorphins, catecholamines, inflammatory cytokines, and endothelins affect nociception and pain perception via G-protein coupled signaling, which is a primary target for many analgesics. (Geppetti et al., 2015; Nourbakhsh et al., 2018) There is evidence from prior studies that some of the identified pathways are involved in chronic pain conditions and pain management. (Chidambaran et al., 2019; Pedersen et al., 2015; Duan et al., 2021; Wang and Burrell, 2018; Hossain et al., 2020; Siuda et al., 2015; Isensee et al., 2017; Gomes et al., 2020; Louwies et al., 2019; Montesino-Goicolea et al., 2020) For instance, Montesino-Goicolea *et al.* reported that enrichment of *GABA Receptor Signaling* pathways by differentially methylated genes between adults chronic pain and healthy controls. (Montesino-Goicolea et al., 2020) Also, enrichment of *GABA Receptor Signaling* pathways have previously been associated with chronic postoperative pain, (Chidambaran et al., 2019) while *Opioid Signaling* and *Endocannabinoid Neuronal Synapse Pathways* have previously been associated with addiction and pain management. (Gomes et al., 2020; Ramesh et al., 2018; Dawley et al., 2017; Regan et al., 2012) Thus, epigenetically induced changes in multiple pathways may play an essential role in cLBP and more significant pain severity in NHBs. These support our findings that racial disparities in non-specific cLBP may be related to epigenetic modification of genes in different pathways signaling pathways. These observations align with prior works suggesting the need to consider the interaction of immune and neurological systems as potential novel targets for pain. (Montesino-Goicolea et al., 2020)

Finally, our stratified race analysis comparing individuals with cLBP against racially similar PFCs suggests the need for race-specific epigenetic studies. One hundred and ten (110) pathways were significantly over-represented in the NHBs compared to 31 pathways in the NHWs group. Nineteen (Fuentes et al., 2007) of the pathways over-represented in NHWs were also over-represented in NHBs, but the p-values were much smaller in NHBs than NHWs. While the p-value is not a perfect predictor of effect size, a smaller p-value between data of equal sample size suggests a more robust effect size. The effect size of the over-represented pathways may be greater in NHBs than NHWs. Also, more genes may be differentially methylated in the NHB than NHW groups. Thus, future epigenetic studies of cLBP should recruit cases and controls from the same racial background.

Strengths and limitations

Our study has several strengths and limitations that must be considered when generalizing the findings. First, to our knowledge, this study provides the first evidence of a potential mechanistic important role for DNAm in racial disparities in cLBP. By using an epigenetic approach, our findings lend themselves to future therapeutic targets to reduce the disparities in cLBP. Also, using racial group-stratified comparisons is a major strength because epigenetic changes are influenced by environmental exposure. Thus, uncovering mechanistic roles requires a within-group comparison to account for differences in distinct lived experiences. This approach indirectly controls for the social nature of the racial group construct and avoids using one racial group as the reference group. Finally, using an epigenomic approach gives novel insight into potential pathways without the limitations of a *priori* knowledge.

There are some limitations to our study. First, despite being the first and largest study to date, our sample size was relatively small. Second, the epigenomic approach gives insight into novel pathways, but the precise mechanism by which DNAm changes affect the signaling pathways and cLBP phenotype need to be confirmed in future studies through the measure of gene expression. It would be highly relevant to investigate the influence of each gene marker on gene expression and cLBP phenotype using targeted sequencing epigenetic approach. Also, some of the racial differences may be due to genetics because genetic polymorphisms can lead to changes in methylation. Third, racial categories in our study were self-identified by the participants. Given that race is a social construct, we believed that using self-identified race would most appropriately capture the potential effects of lived experiences on the epigenome, which may explain the racial disparities in cLBP. Our study did not include a secondary validation of the differentially methylated genes. However, it provides a solid basis for further investigations with larger cohorts and deeper explorations of specific genes or signaling pathways. Additionally, it provides proof-of-concept data addressing the larger issue of how race relates to the risk of chronic diseases in the United States. Finally, the use of blood samples rather than nervous tissues is a limitation. Epigenetic changes are tissue-specific, but DNAm changes in the nervous system have been shown to correlate with changes in blood samples. The use of blood samples provides a readily available marker that can be used clinically. With the use of blood samples, DNAm differences could represent variations in the proportion of cell subtype populations. We will address this in future studies by either directly determining cell population sizes via flow cytometry or single-cell RNAseq or statistically using methods described previously. (Houseman et al., 2012; Jaffe and Irizarry, 2014; Karmaus and Chen, 2017) Finally, we limited this study to individuals with nonspecific cLBP, eliminating many individuals with cLBP (e.g., trauma, rheumatoid arthritis, other chronic pain conditions, etc.). Similarly, while we did an epigenetic race by pain interaction analysis, there was a strong correlation between race and pain severity. Thus, the observed racial differences may be related to pain severity.

Conclusion

In the United States, individuals who self-identify as NHBs report more severe and disabling cLBP compared to NHWs. Even though an individual's self-identified race is not a biological variable, that system of classifying people and associated racism affects virtually every aspect of life, including disease risk. Our findings suggest a potential mechanistic role of epigenetic modifications in racial disparities in cLBP. Specifically, our results indicate that DNAm alterations in pathways involving G-protein coupled receptors, neuronal transmission, and chronic stress may explain some of the variances in racial disparities in cLBP. While future studies are required in larger prospective cohorts with a longitudinal evaluation of DNAm and gene expression with replication of our findings, these results are promising and open novel avenues of epigenetic-based pain disparities research. Given that epigenetic modifications are dynamic and reversible, future targeted epigenetics interventions may help reduce cLBP disparities and improve cLBP management.

Ethics approval and consent of participants

The Institutional Review Board (IRB) at the University of Alabama at Birmingham approved all research included in this study (IRB-170119003). Written informed consent was obtained from all participants under the approval from the IRB.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynpai.2022.100086.

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