



Epigenetic signature of chronic low back pain in human T cells

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Abstract

Objective: Determine if chronic low back pain (LBP) is associated with DNA methylation signatures in human T cells that will reveal novel mechanisms and potential therapeutic targets and explore the feasibility of epigenetic diagnostic markers for pain-related pathophysiology.

Methods: Genome-wide DNA methylation analysis of 850,000 CpG sites in women and men with chronic LBP and pain-free controls was performed. T cells were isolated (discovery cohort, n = 32) and used to identify differentially methylated CpG sites, and gene ontologies and molecular pathways were identified. A polygenic DNA methylation score for LBP was generated in both women and men. Validation was performed in an independent cohort (validation cohort, n = 63) of chronic LBP and healthy controls.

Results: Analysis with the discovery cohort revealed a total of 2,496 and 419 differentially methylated CpGs in women and men, respectively. In women, most of these sites were hypomethylated and enriched in genes with functions in the extracellular matrix, in the immune system (ie, cytokines), or in epigenetic processes. In men, a unique chronic LBP DNA methylation signature was identified characterized by significant enrichment for genes from the major histocompatibility complex. Sex-specific polygenic DNA methylation scores were generated to estimate the pain status of each individual and confirmed in the validation cohort using pyrosequencing.

Conclusion: This study reveals sex-specific DNA methylation signatures in human T cells that discriminates chronic LBP participants from healthy controls.

Keywords: DNA methylation, Low back pain, T cells, Biomarker, Epigenetics

1. Introduction

Chronic pain is debilitating, difficult to treat, and often due to unknown causes. With a life-time prevalence of >80%, low back pain (LBP) is a leading cause of global years lived with disability, with other musculoskeletal conditions, neck pain, and migraine joining in the top 10.²² Therapeutic interventions are often inadequate or associated with undesired consequences; these limitations are amplified by concerns related to opioid misuse.

Roadblocks to the development of nonopioid pain medications include a paucity of validated targets, the lack of diagnostic markers for pain-related pathologies, and a high degree of interindividual variation in response to interventions.

Chronic pain is associated with long-term changes in gene expression that are, at least in part, under epigenetic control.³⁹ Epigenetics refers to modifications that alter gene expression without changing the genetic code. Epigenetic mechanisms

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.painrpts.com).

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PR9 6 (2021) e960

<http://dx.doi.org/10.1097/PR9.0000000000000960>

include DNA methylation, modulation of the chromatin structure, and noncoding RNAs.³⁹ Understanding epigenetic signatures provides insights into underlying disease mechanisms and are emerging as diagnostic biomarkers for complex diseases.³¹ For example, studies have demonstrated broad changes in DNA methylation in blood cells that are sensitive to life experiences including maternal stress, depression, early life abuse, and social economic status in humans^{9,11,35} as well as maternal deprivation in rhesus monkeys.⁴⁰ We and others previously reported chronic pain–related changes in DNA methylation in animal models and in chronic LBP patients,^{1,3,30,44,45} and differential DNA methylation in blood is related to pain sensitivity in healthy subjects.⁸

Chronic pain is associated with the activation of the immune system including increased levels of circulating proinflammatory cytokines, and a role for the adaptative immune system—comprised of B and T cells—is emerging.²⁸ In a proof-of-concept study in rats with peripheral nerve injury, we reported widespread changes in DNA methylation in T cells that partially overlapped with changes in the prefrontal cortex and were predictive of the presence and the severity of neuropathic pain.³⁰

We hypothesize that chronic pain in humans is associated with DNA methylation signatures that will (1) reveal novel therapeutic targets, (2) provide diagnostic markers for pain-related pathophysiology, and (3) offer insights into the high degree of interindividual variability in chronic pain populations. Genome-wide DNA methylation of 850,000 cytosine-guanine CpG sites in T cells from female and male participants with chronic LBP were compared with pain-free controls (discovery cohort, $n = 32$). Differentially methylated CpG sites were identified and used to generate a polygenic methylation score for LBP in women and men. An independent cohort (validation cohort, $n = 63$) of LBP participants and healthy controls was used for validation. Finally, molecular, biological, and cellular functions that are epigenetically dysregulated in individuals living with chronic LBP were identified. These data implicate epigenetic regulation in LBP in humans, identify potential pathological drivers, and raise the potential for clinically useful DNA methylation markers for chronic pain in humans.

2. Methods

2.1. Study design and participants

This study was approved by the Research Ethics Board of the McGill University Health Center (#MP-CUSM-13-402). To minimize selection bias, participants were recruited from community advertisements and all eligible participants were accepted. Participation consisted of a single visit to the Montreal General Hospital (recruitment from 2016 to 2019) during which subjects completed questionnaires and a blood sample was collected. This study consisted of 2 cohorts (**Table 1**): the discovery cohort ($n = 32$; women: 8 controls and 8 LBP; men: 8 controls and 8 LBP) and the validation cohort ($n = 63$; women: 13 controls and 19 LBP; men: 13 controls and 18 LBP). The discovery cohort was selected from the first 75 participants and was intentionally balanced for age and sex. No formal sample size analysis was performed a priori because no data were available on T-cell DNA methylation in patients with chronic LBP. However, given the actual average methylation variance of 3%, $n = 8$ would enable the detection of differences of 10% or greater at individual CpG at $\alpha = 0.05$ and power of 0.8 after correcting for multiple comparisons. Inclusion criteria for LBP included self-reported LBP for at least 1 year with an average daily pain score of at least 3/10. Etiology was not addressed in this study. Exclusion criteria

for all participants were body mass index >35 , pregnancy, age outside of 25 to 65, presence of other chronic pain conditions, and previous back surgery.

2.2. Self-reported measures

Questionnaires included the Canadian adaptation of the NIH Low Back Pain Taskforce minimum recommended data set and standardized, validated questionnaires measuring neuropathic pain (*DN4*), global health (*EQ-5D-5L*), disability (*ODI2.1a*), somatization (*SCL90R*, *somatization subscale*), mood (*HADS*), pain catastrophizing (*PCS*), and a Self-Administered Comorbidity Questionnaire.³⁷ Participant characteristics (**Table 1**) are expressed as mean \pm SEM or as percentage (%). As pain intensity, disability, depressive symptoms, global health, and somatization do not follow normal distribution ($P < 0.05$ in the Kolmogorov–Smirnov test), the Mann–Whitney *U* test was used. For the other variables with a normal distribution, unpaired *t* tests were used to compare control and LBP participants using GraphPad Prism and changes were considered significant if $P < 0.05$. Categorical variables were analyzed using the Fisher exact test.

2.3. T-cell isolation, DNA extraction, and bisulfite conversion

CD3⁺ T cells were isolated from 8 mL blood collected in EDTA (Ethylenediaminetetraacetic acid) tubes, and leukocytes were freshly isolated using Ficoll gradient separation. T cells were positively isolated using Dynabeads CD3 isolation kit (Invitrogen), and pellets were immediately frozen at -80°C . DNA was isolated using AllPrep DNA/RNA Mini Kit (Qiagen, Canada). Bisulfite conversion was performed (EZ-DNA methylation Gold Kit; Zymo Research, Irvine, CA), and DNA methylation profiles were generated by Genome Quebec according to standard protocols using Illumina 850 K bead arrays (Infinium MethylationEPIC Kit).

2.4. DNA methylation data processing

Illumina arrays were analyzed using the ChAMP Bioconductor package in R.⁴⁷ **Figure 1** illustrates the different steps of the analysis. Samples were randomized on arrays to mitigate batch effects. In brief, IDAT files were imported and filtered with `champ.load()` with default settings. Men and women were analyzed separately, and remaining probes were normalized with `champ.norm()` using the BMIQ (Beta Mixture Quantile dilation) method. Batch effects from slides were corrected with `champ.runCombat()`. Differentially methylated positions (DMPs) were obtained using `champ.DMP()` (Benjamini–Hochberg FDR correction). Differential methylation was calculated as the ratio (β -value) of intensities between methylated cytosines at each CpG site and total locus signal intensity.

The data have been deposited in NCBI's Gene Expression Omnibus (GEO)¹⁹ and are accessible through GEO Series accession number GSE 162350.

2.5. Identification of a polygenic methylation score

Using the discovery cohort, a polygenic methylation score was developed in women and men as a proof-of-concept potential biomarker for LBP. Polygenic scores are used in cases when one single CpG site cannot achieve the desired significance.²⁶ A Cohen score was calculated for each probe to shortlist probes with the highest effect size (Cohen $D > 2$) then shortlisted by the percentage of differential methylation. Top probes were

Table 1
Demographic and clinical characteristics of controls and low back pain participants in the (A) discovery and (B) validation cohorts*.

Parameters	Groups					
	Control women	LBP women	P	Control men	LBP men	P
(A) Discovery cohort						
Number	8	8	—	8	8	—
Age	43.8 ± 4.6	41.3 ± 3.8	n.s.	43.8 ± 4.0	42.6 ± 3.6	n.s.
Height	162.1 ± 2.0	162.9 ± 2.4	n.s.	178.1 ± 1.7	176.8 ± 2.4	n.s.
Weight	64.1 ± 3.5	71.8 ± 5.1	n.s.	79.9 ± 4.3	81.8 ± 2.4	n.s.
BMI	24.4 ± 1.4	26.9 ± 1.5	n.s.	25.2 ± 1.3	26.2 ± 0.5	n.s.
Pain intensity (0-10)	0 ± 0	5.6 ± 0.6	P < 0.001	0.3 (0.3)	5.4 (0.8)	P < 0.001
Pain duration (%)						
1–5 y	—	50	—	—	62.5	—
More than 5 y	—	50	—	—	37.5	—
Living condition (%)						
Alone	12.5	12.5	n.s.	62.5	62.5	n.s.
Not alone	87.5	87.5	n.s.	37.5	37.5	n.s.
Race (%)						
White	87.5	62.5	n.s.	100	62.5	n.s.
Asian or Pacific Islander	12.5	0	n.s.	0	12.5	n.s.
Black	0	37.5	n.s.	0	12.5	n.s.
Others	0	0	n.s.	0	12.5	n.s.
Education (%)						
Less than high school	0	0	n.s.	0	0	n.s.
High school	0	0	n.s.	0	0	n.s.
College, certificate	37.5	62.5	n.s.	25	25	n.s.
Bachelor's degree	25	12.5	n.s.	37.5	12.5	n.s.
Master's degree, certificate above bachelor	12.5	25	n.s.	12.5	50	n.s.
Higher than master's degree	25	0	n.s.	25	12.5	n.s.
ODI (/50)	0.1 ± 0.1	9.1 ± 1.7	P < 0.001	0.4 ± 0.4	6.5 ± 1.5	P < 0.001
Anxiety score (/21)	3.1 ± 0.6	4.9 ± 1.6	n.s.	2.3 ± 1.1	5.5 ± 0.8	P < 0.05
Depression score (/21)	0.8 ± 0.4	3.8 ± 1.1	P < 0.05	0.8 ± 0.5	3.5 ± 0.6	P < 0.01
DN4 (/7)	0.0 ± 0.0	2.8 ± 0.8	P < 0.01	0.0 ± 0.0	2.3 ± 0.8	P < 0.01
EQ-5D-5L (/25)	5.0 ± 0.0	8.9 ± 0.7	P < 0.001	5.1 ± 0.1	8.1 ± 0.5	P < 0.001
SCL-90-R (/48)	1.3 ± 0.3	7.3 ± 1.6	P < 0.01	1.1 ± 0.7	7.0 ± 1.7	P < 0.01
PCS (/52)	5.4 ± 2.5	15.3 ± 3.7	P < 0.05	6.1 ± 3.1	12.9 ± 4.0	n.s.
(B) Validation cohort						
Number	13	19	—	13	18	—
Age	38.5 ± 3.5	46.1 ± 2.7	n.s.	43.1 ± 3.2	48.4 ± 2.6	n.s.
Height	165.5 ± 1.7	163.2 ± 1.7	n.s.	175.5 ± 2.2	175.8 ± 1.9	n.s.
Weight	64.6 ± 2.9	64.1 ± 2.6	n.s.	77.5 ± 3.4	84.2 ± 2.5	n.s.
BMI	23.6 ± 1.0	23.9 ± 0.9	n.s.	25.1 ± 0.9	27.3 ± 0.7	n.s.
Pain intensity (0-10)	0.1 ± 0.1	6.9 ± 0.5	P < 0.001	0 (0)	6.6 (0.4)	P < 0.0001
Pain duration (%)						
1–5 y	—	47.4	—	—	44.4	—
More than 5 y	—	52.6	—	—	55.6	—
Living condition (%)						
Alone	23.1	15.8	n.s.	53.8	27.8	n.s.
Not alone	76.9	84.2	n.s.	46.2	72.2	n.s.
Race (%)						
White	46.1	68.4	n.s.	69.2	44.45	n.s.
Asian or Pacific Islander	15.4	5.3	n.s.	15.4	0	n.s.
Black	15.4	15.8	n.s.	0	11.1	n.s.
Others	23.1	10.5	n.s.	15.4	44.45	n.s.
Education (%)						
Less than high school	0	0	n.s.	0	0	n.s.
High school	0	10.5	n.s.	7.7	22.2	n.s.
College, certificate	15.4	42.1	n.s.	30.8	27.8	n.s.
Bachelor's degree	15.4	15.8	n.s.	38.4	22.2	n.s.
Master's degree certificate above bachelor	53.8	21.1	n.s.	7.7	27.8	n.s.
Higher than master's degree	15.4	10.5	n.s.	15.4	0	n.s.

(continued on next page)

Table 1 (continued)**Demographic and clinical characteristics of controls and low back pain participants in the (A) discovery and (B) validation cohorts*.**

Parameters (B) Validation cohort	Groups					
	Control women	LBP women	P	Control men	LBP men	P
ODI (/50)	0.5 ± 0.3	14.2 ± 1.2	<i>P</i> < 0.001	0.1 ± 0.1	11.1 ± 1.3	<i>P</i> < 0.0001
Anxiety score (/21)	2.5 ± 0.7	8.9 ± 1.0	<i>P</i> < 0.001	3.1 ± 0.7	7.3 ± 0.9	<i>P</i> < 0.01
Depression score (/21)	1.6 ± 0.8	6.6 ± 0.9	<i>P</i> < 0.0001	1.5 ± 0.6	5.9 ± 1.0	<i>P</i> < 0.0001
DN4 (/7)	0.0 ± 0.0	3.2 ± 0.5	<i>P</i> < 0.0001	0.0 ± 0.0	2.3 ± 0.4	<i>P</i> < 0.0001
EQ-5D-5L (/25)	5.3 ± 0.2	10.6 ± 0.8	<i>P</i> < 0.0001	5.2 ± 0.1	9.6 ± 0.5	<i>P</i> < 0.0001
SCL-90-R (/48)	1.5 ± 0.4	13.5 ± 1.9	<i>P</i> < 0.0001	0.3 ± 0.2	9.9 ± 1.6	<i>P</i> < 0.0001
PCS (/52)	7.8 ± 2.2	25.8 ± 3.2	<i>P</i> < 0.01	3.1 ± 1.4	19.2 ± 2.8	<i>P</i> < 0.0001

Categorical variables were analyzed using the Fisher exact test. Continuous variables are expressed as mean ± SEM, and an unpaired *t*-test or the Mann-Whitney *U*-test was used to compare control and LBP participants. BMI, body mass index; DN4, Douleur Neuropathique 4; EQ, EuroQol; LBP, low back pain; ODI, Oswestry Disability Index; PCS, Pain Catastrophizing Scale; SCL, symptoms checklist.

subjected to penalized regression and a set of 3 probes was identified. For each individual, the combined weight of these 3 probes based on the regression coefficient was used to calculate a polygenic methylation score as a single variable that measures a sample's membership in either the control or the LBP group (0 for control and 1 for LBP).

Female Polygenic Methylation Score = $(\beta_1 \times (-1.77) + \beta_2 \times 2.46 + \beta_3 \times (-1.36)) - 1.32$ (β_1 = methylation level of cg26114124, β_2 = cg07420274, β_3 = cg20331269).

Male Polygenic Methylation Score = $(\beta_1 \times (-1.98) + \beta_2 \times 0.23 + \beta_3 \times (-2.92)) + 2.89$ (β_1 = methylation level of cg21149944, β_2 = cg22831726, β_3 = cg25393494).

Two-way analysis of variance was used to compare methylation scores (women vs men, control vs LBP) followed by a Sidak test for multiple comparisons.

2.6. Validation using pyrosequencing and an independent cohort

T cells were collected and processed as described above from participants in the validation cohort. Pyrosequencing of selected genes was performed on all participants (discovery and validation cohorts); primers are listed in Supplementary Table 1 (available at <http://links.lww.com/PR9/A131>). PCR amplification and pyrosequencing were performed using standard methods.¹² In brief, biotinylated PCR products were incubated with Streptavidin Sepharose beads (GE Healthcare, Mississauga, Canada), followed by denaturation. Pyrosequencing was performed using PyroMark Q24 and analyzed with PyroMark Q24 Software (Qiagen, Toronto, ON, Canada). Data were expressed as mean ± SEM and analyzed by the Student *t* test. Owing to poor sample quality or quantity, pyrosequencing data for 4 women and 4 men are missing.

Statistical analyses were performed using logistic regression at CpG sites validated with pyrosequencing to evaluate the relationship between CpG methylation value (independent variable) and chronic pain status (dependent variable). There was no age effect, and adding age as a covariate did not change the results.

2.7. Genome-wide identification of differentially methylated positions

Genome-wide methylation analysis was performed with the Montreal Clinical Research Institute for quality control, bioinformatics, and statistical analysis. As no significant DMPs were found using adjusted *P*-value < 0.05, DMPs were defined with a nominal *P*-value < 0.05 and a $(\Delta\beta) \geq 10\%$ between the 2 groups.

After log₂ transformation, β values were then mean and centered for heatmap representation with hierarchical clustering applied on probes and samples using the complete linkage method. A subset analysis was performed on 2 distinct subclusters of LBP and control women (*n* = 4 each). For this analysis, DMPs were defined with adjusted *P*-value < 0.01 and a $(\Delta\beta) \geq 20\%$.

Differences in the genomic distribution of the DMPs in both women and men and between hypomethylated and hypermethylated DMPs were assessed using a Fisher exact test on DMP feature (eg, TSS200, TSS1500, ...) as extracted from the Infinium MethylationEPIC v1.0 B4 Manifest.

To assess the overlap between DMPs in our study and publicly available data, Venn diagrams were constructed.⁷ To compare results between human, mouse, and rat, BioMart database was used to identify human gene orthologs. A hypergeometric test was used to test for enrichment of LBP-related genes identified here with the public databases.

2.8. Functional enrichment analysis

Gene Ontology (GO) and pathway enrichment analysis were performed on DMPs using g:Profiler with g:SCS multiple testing correction method.⁴¹ Enriched GO terms were categorized into molecular function, biological processes, and cellular components. Enriched GO terms and KEGG pathways with an adjusted *P*-value < 0.05 are represented as enriched scores expressed as $-\log_{10}$ (adjusted *P*-value). Enriched KEGG maps were drawn with Pathview Web.

3. Results

3.1. Differential methylation analysis in the discovery cohort

Of the 736,414 CpGs identified in men, 179 were hypermethylated and 240 were hypomethylated. Of the 735,863 CpGs identified in women, 601 were hypermethylated and 1895 were hypomethylated (*P*-value < 0.05 and a $(\Delta\beta) \geq 10\%$, **Figure 2A** and Supplementary Table 2, available at <http://links.lww.com/PR9/A131>). In the subset of women analyzed separately, 950 hypermethylated and 8750 hypomethylated CpGs were found (adjusted *P*-value < 0.01 and a $(\Delta\beta) \geq 20\%$, Supplementary Figure 1A and Supplementary Table 3, available at <http://links.lww.com/PR9/A131>). Using the discovery cohort, we calculated a polygenic methylation score from circulating T cells that requires only 3 CpGs sites (**Fig. 2B**) to predict the pain status of each individual (0 = no pain and 1 = pain, **Fig. 2C**). The individual CpGs and the composite methylation scores were sex specific. In men, 2 of the CpGs in the composite score correspond to the same gene, ZNF718.

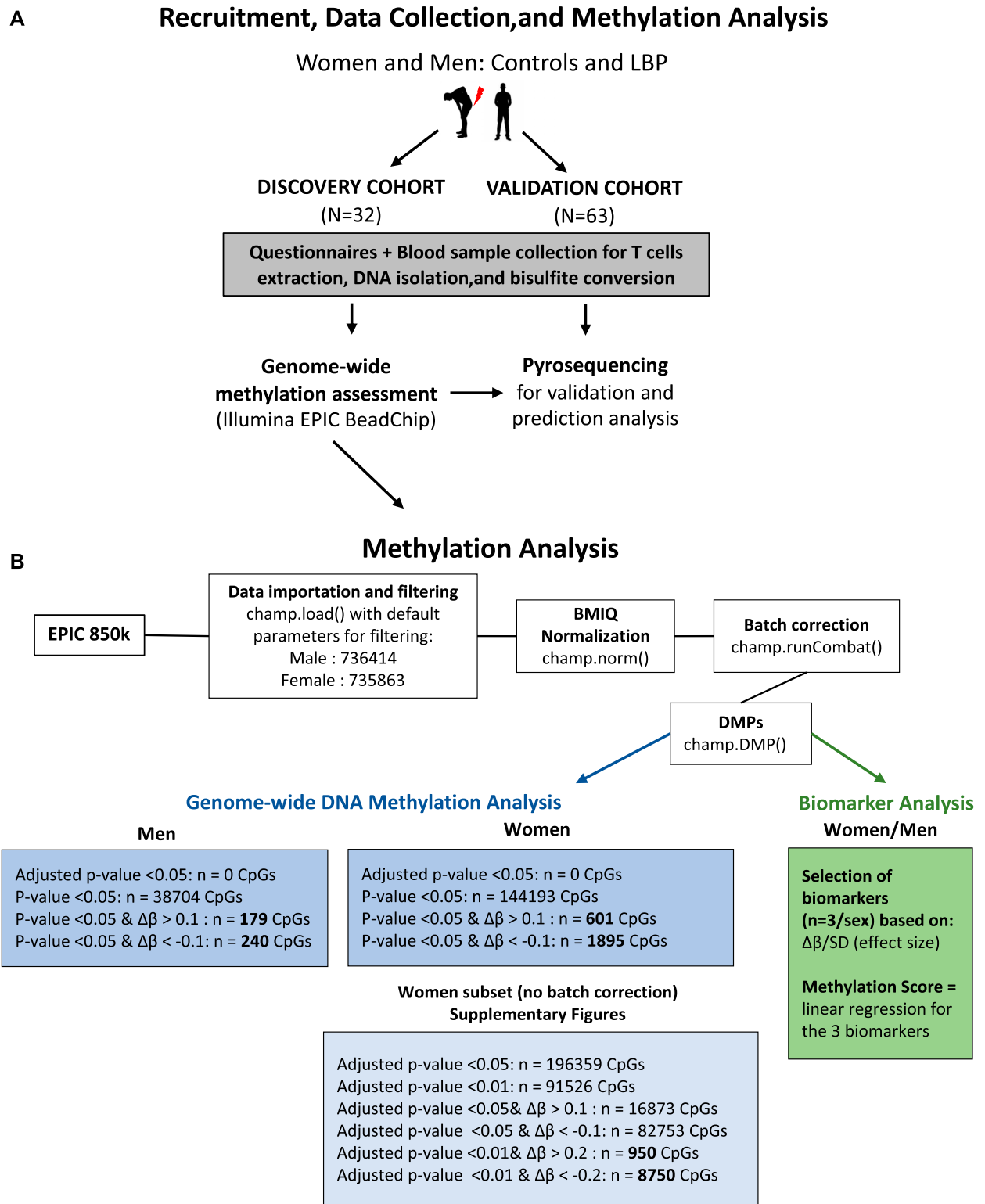


Figure 1. Overview of the study design including (A) the recruitment, data collection, and analysis, as well as (B) the flowchart for the genome-wide methylation analysis study*. *LBP, low back pain; $\Delta\beta$, absolute difference in methylation value between control and LBP groups.

3.2. Confirmation of differential methylation using pyrosequencing and the validation cohort

To validate results from the array-based analysis, probes covering 3 DMPs from the polygenic methylation score's analysis (cg07420274 for women and cg21149944 and cg22831726 for men) were selected for measurement of DNA

methylation at single-nucleotide resolution using pyrosequencing (Fig. 3). In the discovery cohort, the differences between LBP and controls obtained with Illumina were confirmed with pyrosequencing in both women and men (Fig. 3A, B, respectively). In the independent validation cohort, both DMPs of interest in men were validated by pyrosequencing. Although

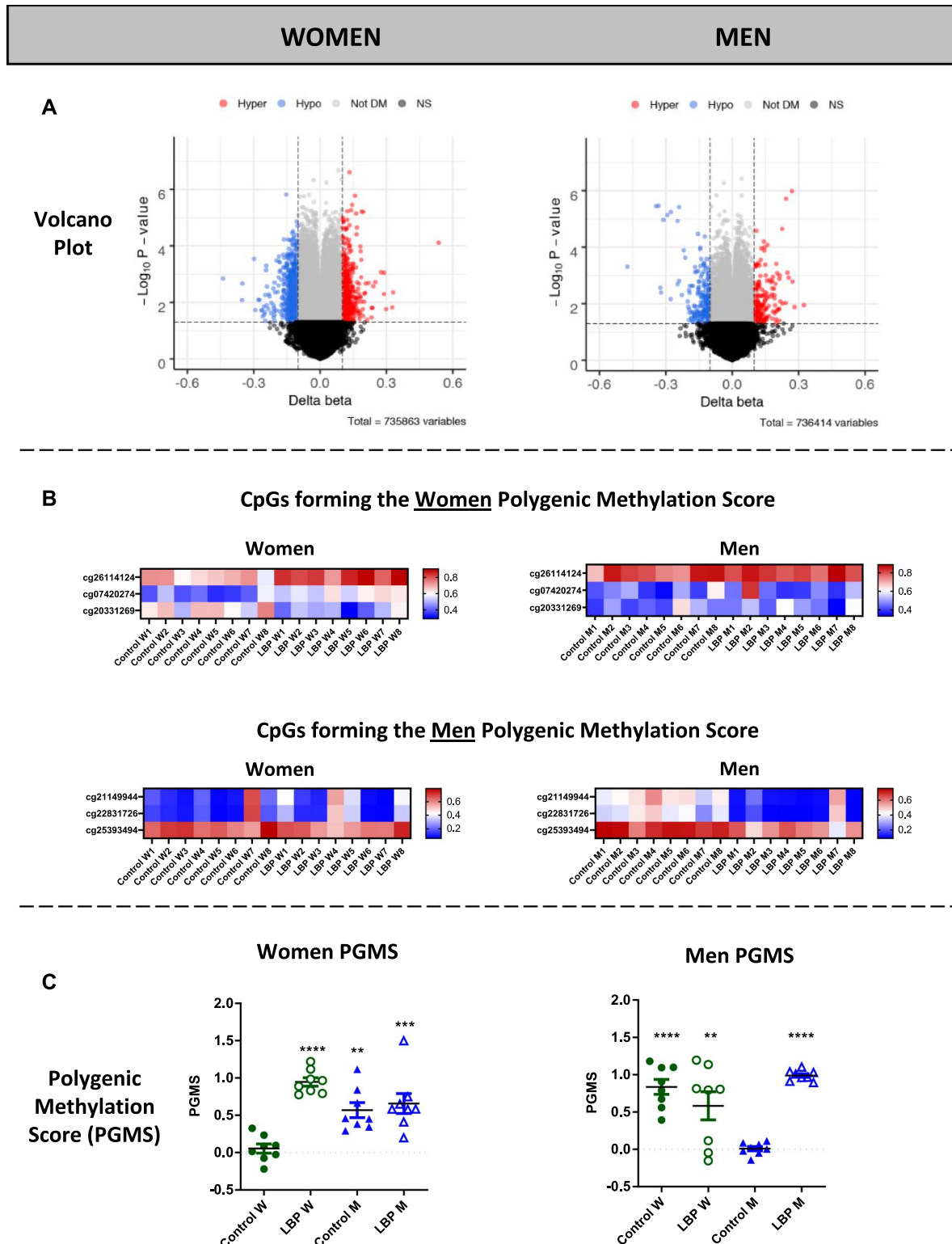


Figure 2. (A) Volcano plots of the differentially methylated positions with x and y axes displaying, respectively, the delta beta values (effect size) and the log₁₀ of the *P* values for each CpGs site. Hypermethylated and hypomethylated CpGs in LBP vs controls (delta beta > 10% and *P* < 0.05) are represented in red and blue, respectively. (B) Heatmap representation of the methylation profile of the selected CpGs in both women and men used to construct the (C) polygenic methylation score. Two-way analysis of variance (ANOVA) followed by Sidak multiple comparisons, ** = *P* < 0.01, *** = *P* < 0.001, **** = *P* < 0.0001. *LBP, low back pain; Not DM = not differentially methylated, NS, no significant; W, women; M, men.

differential methylation of cg07420274 in women was not statistically significant by pyrosequencing (*P* = 0.07), the direction of change was consistent with the array-based

analysis. Results from the array-based analysis correlated with pyrosequencing data (*r* = 0.44, *P* < 0.05, Supplementary Figure 2, available at <http://links.lww.com/PR9/A131>).

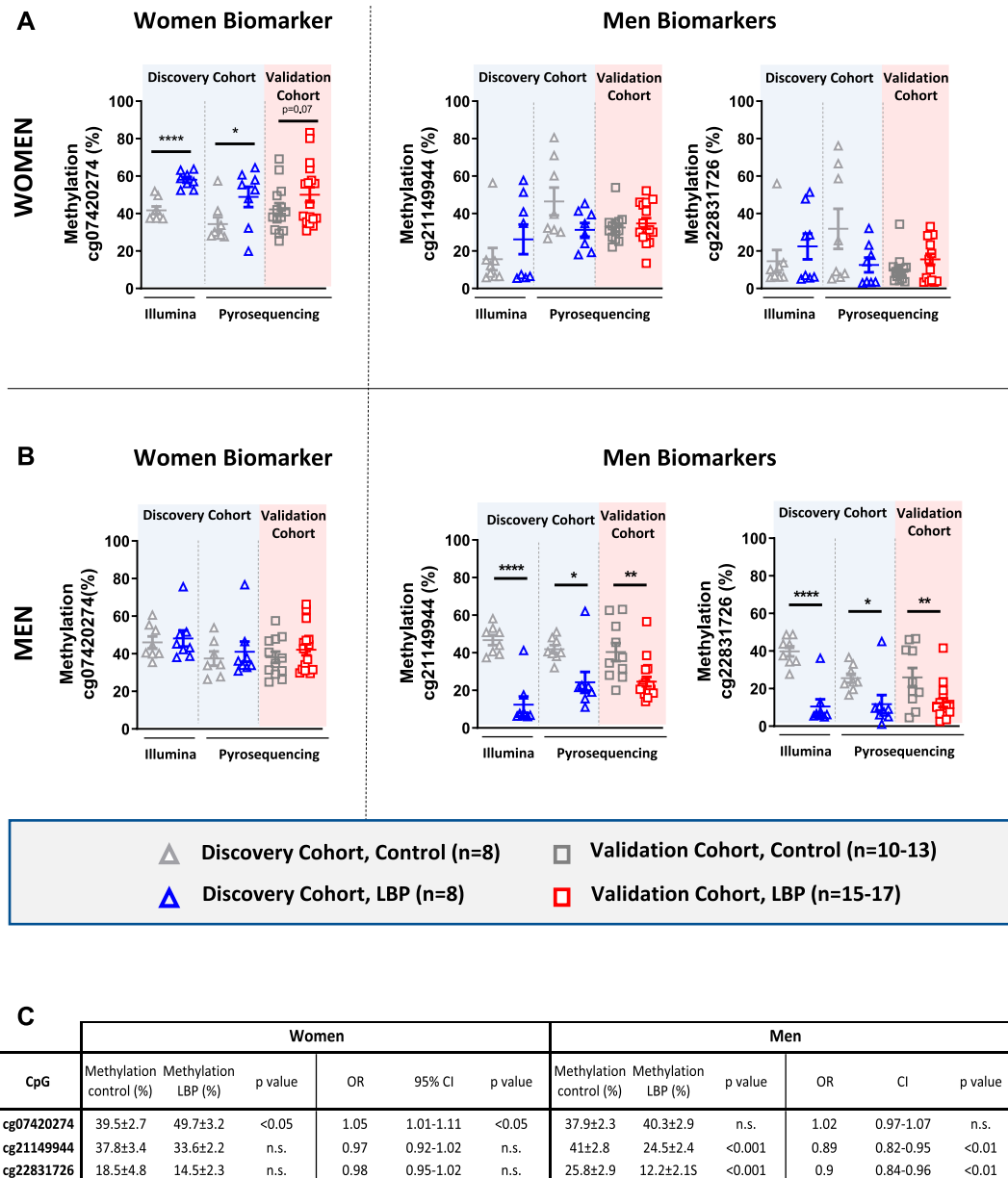


Figure 3. Percentage of methylation using Illumina (850K Epic Array) or targeted pyrosequencing of the 3 CpGs selected for validation in the discovery and validation cohorts in (A) women and (B) men. Association of the 3 CpGs methylation with the risk of having chronic low back pain using a logistic regression (C). Unpaired two-tailed (discovery cohort) or one-tailed (validation cohort) *t* test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

3.3. Association between methylation status and probability of having chronic low back pain

In women, the percentage of methylation at position cg07420274 was $39.5 \pm 2.7\%$ and $49.7 \pm 3.2\%$ in control ($n = 21$) and LBP groups ($n = 25$), respectively ($P < 0.05$, **Fig. 3C**). A statistically significant association between methylation at cg07420274 and LBP was observed (OR = 1.05, 95% CI: 1.01–1.11, $P = 0.03$).

In men, a statistically significant association was found between LBP and cg21149944 methylation (OR = 0.89, 95% CI: 0.82–0.95, $P = 0.0015$) as well as cg22831726 methylation (OR = 0.9, 95% CI: 0.84–0.96, $P = 0.0036$, **Fig. 3C**).

3.4. Distribution analysis of differentially methylated positions

Although DNA methylation within the transcription start site (TSS) is often predicted to silence the respective gene, the impact of methylation in other genomic regions is less clear. The distribution of hypermethylated and hypomethylated DMPs was therefore investigated per genetic feature.

Overall, 75.9% of DMPs in women and 57.3% in men were hypomethylated (**Fig. 4A**). Statistically significant differences between the proportions of hypermethylated vs hypomethylated DMPs were observed for the TSS1500 and TSS200, the

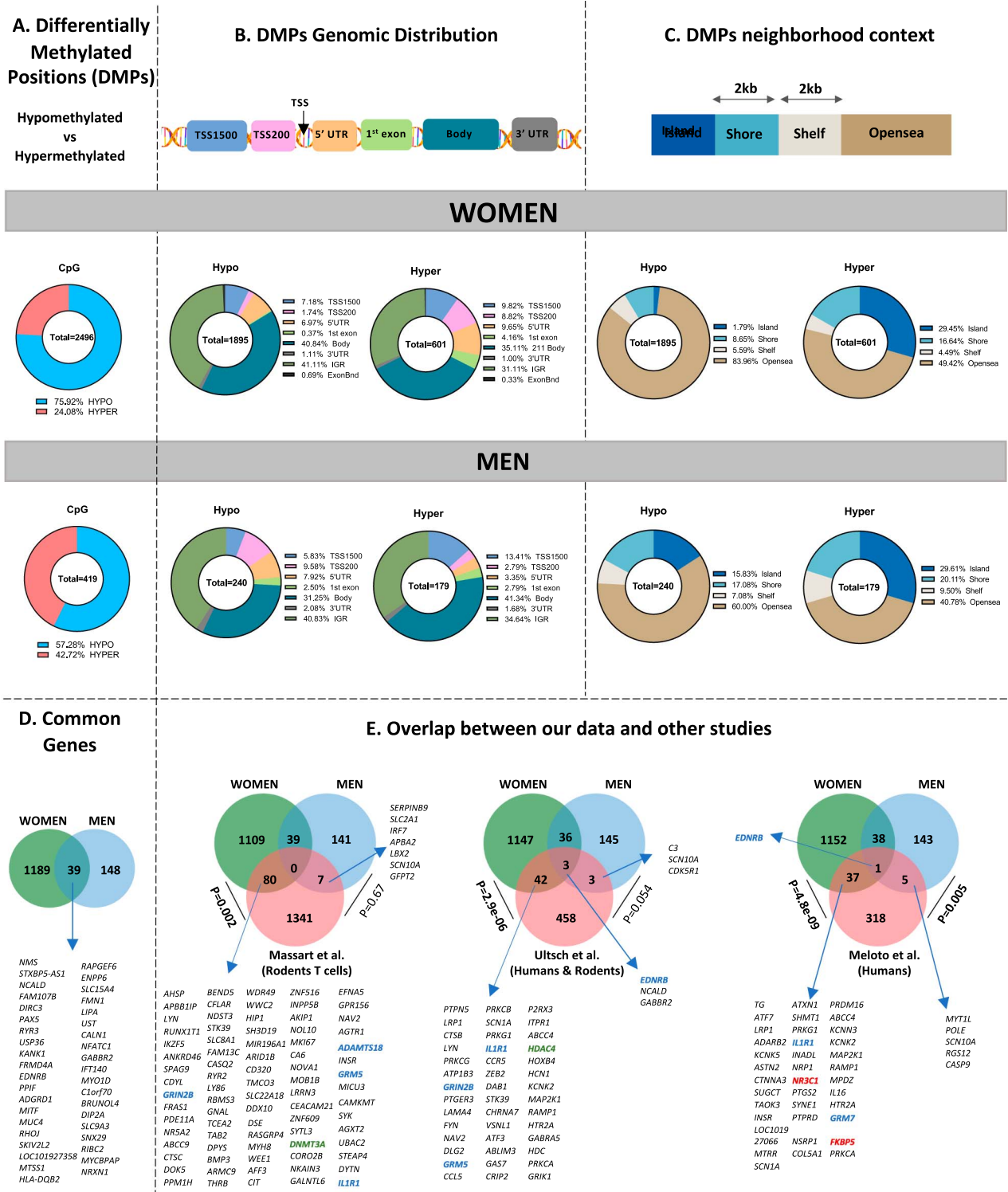


Figure 4. Characteristics of the differentially methylated positions (DMPs) between healthy controls and LBP participants*. Representation of (A) the hypermethylated and hypomethylated positions, (B) the genomic distribution, and (C) the neighborhood context of the DMPs detected in LBP participants compared with controls. Venn diagrams representing (D) the common differentially methylated genes between women and men in our study and (E) the overlap between our data and other studies on rat T cells (Massart et al), human pain genes (Meloto et al.), and rodent pain-related genes (Ultsh et al). For overlaps, pain, stress, and epigenetic-related genes mentioned in the text were highlighted in blue, red, and green, respectively. Significance of overlap between 2 groups was determined using the hypergeometric test. *LBP, low back pain.

5' untranslated region (5'UTR), the gene body, the intergenic region, and the first exon (women only) (Fig. 4B and Supplementary Table 4, available at <http://links.lww.com/>

PR9/A131). The same profile was observed in the subset analysis of women (Supplementary Figure 1B, available at <http://links.lww.com/PR9/A131>). Interestingly, although most

DMPs in women were hypomethylated, only a small percentage (1.79%) were in CpG islands, compared with 29% of all hypermethylated CpGs in the islands (**Fig. 4C**).

3.5. Overlaps between women and men, humans and rodents, and previously identified pain-relevant genes

Women and men had 39 differentially methylated genes in common (**Fig. 4D**). Genes that were differentially methylated in both this study and our previous epigenome-wide rodent study are shown in **Figure 4E**, with a significant enrichment between rat T cells after nerve injury³⁰ and female human LBP genes (80 genes, $P = 0.002$).

We compared our current findings with pain literature (The Pain Genes Database: Ultsch et al.; <http://www.jbldesign.com/jmogil/enter.html> and^{27,48} The Human Pain Genetics Database; <http://links.lww.com/PAIN/A520>); common³² genes are shown in **Figure 4E**. Significant enrichment between women in our study and the Pain Genes Database were observed (42 genes, $P = 9.9e-06$). Significant enrichment was also found for both women and men in our study and the Human Pain Genetics Database (37 genes, $P = 4.8e-09$ and 5 genes, $P = 0.005$, respectively).

3.6. Broad signature of DNA methylation in chronic low back pain

Methylation analysis in women revealed 2496 DMPs between controls and chronic LBP participants with partial separation between groups (**Fig. 5A**). In males, clustering analysis for the 419 DMPs between controls and LBP participants generated complete separation between groups (**Fig. 5B**). The top 10 hypermethylated and hypomethylated DMPs for both women and men are listed (**Fig. 5C, D**, respectively) as well as for the subset group of women (Supplementary Figure 1D, available at <http://links.lww.com/PR9/A131>). In men, 7 of the 10 most hypomethylated DMPs correspond to the gene ZNF718.

Analysis of the clusters within the female LBP participants (Supplementary Figure 1C, available at <http://links.lww.com/PR9/A131>) revealed differences in clinical presentation (Supplementary Figure 3, available at <http://links.lww.com/PR9/A131>). Specifically, although pain intensity was similar in all LBP women ($P = 0.85$), those belonging to the highly clustered group showed significant increases in disability (ODI, $P = 0.004$) and depression (HADS, $P = 0.03$) compared with the other LBP participants. This suggests it may be possible to link methylation signatures to clinical subgroups of LBP patients.

3.7. Gene Ontology enrichment analysis

To gain functional insights, the list of gene-annotated DMPs was subjected to Gene Ontology (GO) enrichment analysis (Supplementary Table 5, available at <http://links.lww.com/PR9/A131>).

In women, the top 10 GO terms categorized into molecular function, biological processes, and cellular component are illustrated in **Figure 5E**. Cell junction (GO:0030054; adj-p = 4.9×10^{-10}), cell periphery (GO:0071944; adj-p = 5.6×10^{-10}), and development process (GO:0032502; adj-p = 8.1×10^{-9}) had the highest enrichment scores. Different methylation of known epigenetic regulators including DNA methyltransferases (DNMT3a) and histone deacetylase (HDAC4 and HDAC11) were identified in this analysis. Enrichment analysis for the subset group of women is illustrated in Supplementary Figure 4A, available at <http://links.lww.com/PR9/A131>.

In men, analysis revealed only 4 enrichment GO terms (**Fig. 5F**), all belonging to the cellular component, the first 2 were MHC class II protein complex and MHC protein complex, representing the major histocompatibility complex that plays a central role in the immune system.

For both woman and men, GO enrichment analysis was also performed on differentially hypermethylated and hypomethylated CpGs separately (Supplementary Figures 5 and Supplementary Table 5, available at <http://links.lww.com/PR9/A131>).

3.8. Pathway enrichment analysis

To generate further insight into functional pathways, we also performed KEGG pathway annotation. In women, 4 enriched pathways were found (**Fig. 5G**): calcium signaling, glutamatergic synapse, axon guidance, and Rap1 signaling pathway (Supplementary Figure 6, available at <http://links.lww.com/PR9/A131>). When pathway analysis was performed for hypermethylated or hypomethylated DMPs separately, the hypomethylated CpGs generated similar results to the grouped analysis (Supplementary Figure 5C, available at <http://links.lww.com/PR9/A131>). Similar pathways were also observed in women subset analysis (Supplementary Figure 4B, available at <http://links.lww.com/PR9/A131>). No significant pathway enrichment was found in men.

4. Discussion

Epigenetic programming provides a mechanism by which previous life experience influences our response to new challenges. These challenges (eg, disease onset and physical or psychological stress), in turn, embed maladaptive states by reprogramming the epigenome. In contrast to genetics, epigenetics is dynamically regulated and can be modulated therapeutically.^{24,36}

We examined the association between LBP and epigenome-wide DNA methylation in T cells isolated from patients and pain-free controls. Key observations include divergent epigenetic signatures of LBP in men and women, proof-of-concept evidence for epigenetic pain biomarkers in lymphocytes and identification of individual genes and gene families with a possible role in LBP pathology. Understanding the role of epigenetics in health and disease in general and chronic pain in particular may generate insights into new therapeutic targets, provide diagnostic markers for risk and recovery, and deliver insights into interindividual differences to support personalized medical approaches.

Chronic LBP is a complex and multifactorial disease to which both genetic and environmental factors contribute.²¹ Many environmental factors, such as early trauma, low socioeconomic status, or depression, are mediated, in part, by long-term epigenetic reprogramming.^{9,11,35} Using reduced representation bisulfite sequencing, Aroke et al. demonstrated differential methylation in the whole blood between chronic LBP participants and pain-free controls.⁴ This study adds to other reports linking DNA methylation in human peripheral blood to chronic pain, including postsurgical pain,^{13,14} fibromyalgia,¹⁵ chronic widespread musculoskeletal pain,²⁹ and neuropathic pain.⁴⁶

We identified a polygenic DNA methylation score from circulating T cells that requires only 3 DMPs to measure the pain status of each individual; several of these were validated in an independent cohort using pyrosequencing. The DMPs and scores were sex specific. This is consistent with the growing literature, suggesting sex-specific effects in pain-induced epigenetic changes,²³ in the immune component of pain,⁴² and in

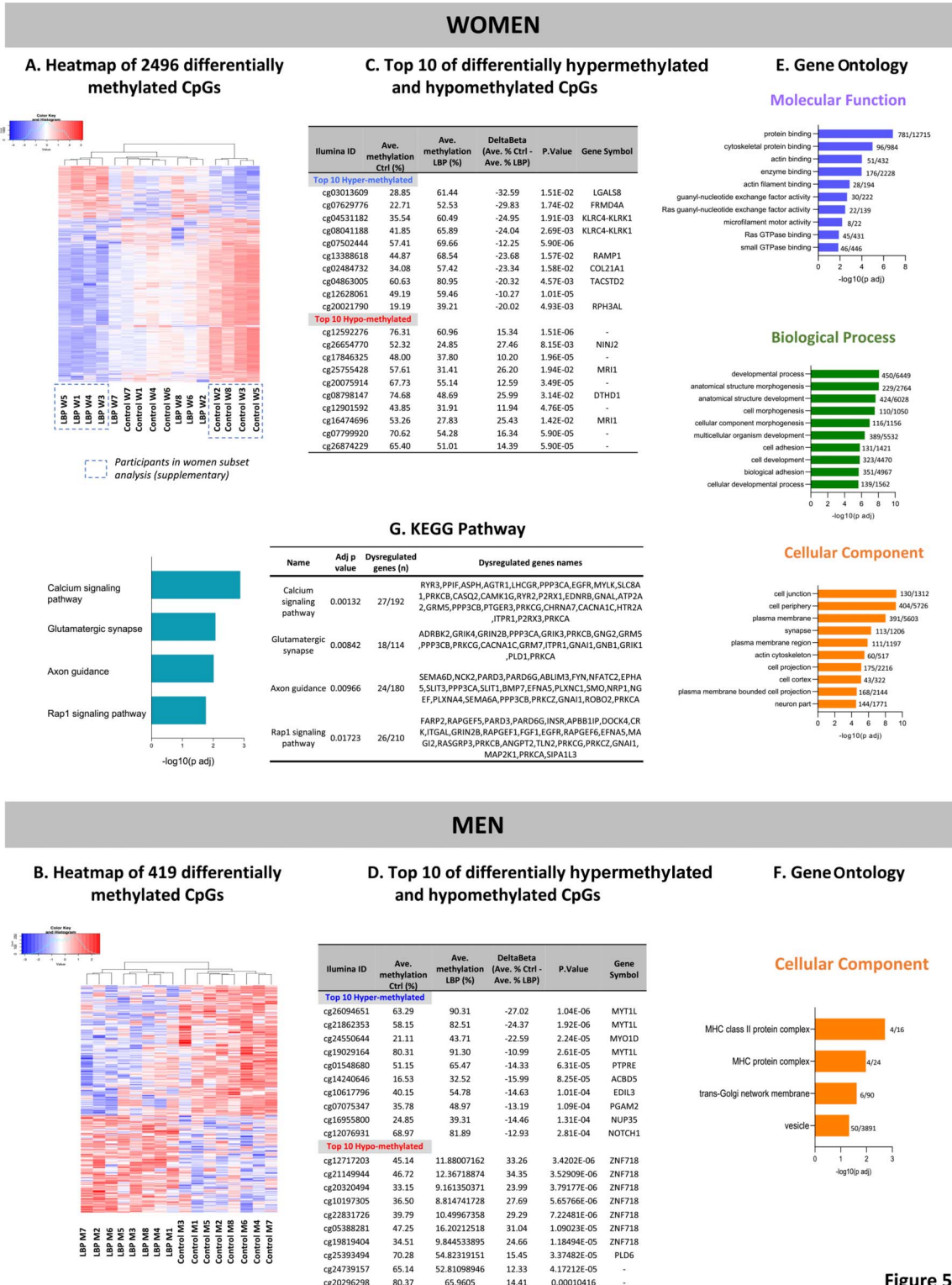


Figure 5

Figure 5. Heatmaps showing methylation signatures of (A) 2496 CpGs sites in women and (B) 419 in men. B values (after log₂ transformation) are depicted using a red (hypermethylated in LBP) to blue (hypomethylated in LBP) methylation gradient. The top 10 differentially hypermethylated and hypomethylated CpGs are listed in both women (C) and men (D). Finally, the top 10 enriched Gene Ontology terms and KEGG pathways are shown in women (E and G) and men (F). Data are presented as enriched scores expressed as $-\log_{10}$ (adjusted *P*-value) with adj *P*-value < 0.05. LBP, low back pain.

stress responses.⁶ Thus, although LBP affects both sexes,³³ our data revealed striking sex differences in DNA methylation signatures, suggesting fundamentally different underlying mechanisms and the possibility of sex-specific epigenetic biomarkers and therapeutic approaches.

In women with LBP, we identified 2496 CpGs that met our criteria for differential methylation, more than 3 quarters being hypomethylated. This persisted in promoter regions (TSS200 and 1500) where hypomethylated CpGs represented 60% of the total changes. This is consistent with findings in women with interstitial

cystitis or bladder pain syndrome, where DNA methylation analysis of pelleted urine sediment showed decreased methylation in multiple CpGs in the MAPK pathway.¹⁰ In utopic endometrial tissue samples from patients with severe endometriosis, interrogated CpG sites were also hypomethylated.²⁵

In women, Gene Ontology enrichment analysis revealed DMPs associated with processes at the cellular and molecular levels including those involved in cell junction, cell periphery, and development. Some of the genes in the enriched ontologies include pain genes (*EGFR*, *GRIN2B*, *GRM5*, and *GAD1*), genes involved in the extracellular matrix (ECM) such as proteases (eg, *MMP15*, *ADAMTS18*, and *ADAMTS16*), collagen (*COL12A1*), glycoproteins (*LAMA1*), integrins (*ITGB6*), and growth factors (eg, *BMP3*, *BMP7*, and *TGFBR2*), interleukins and their receptors (eg, *IL1R1*), and genes associated with epigenetic processes (eg, *HDAC4*, *HDAC11*, and *DNMT3a*). Although LBP is multifactorial, intervertebral disc degeneration is a potential underlying cause and is associated with altered ECM turnover and inflammation.⁵ A recent study suggesting a role for ECM reorganization in the nervous system in chronic pain.³⁸ Furthermore, the changes observed in the current study may be secondary to LBP but rather reflect the unrelenting stress of living with chronic pain. This is supported by the overlap with *The Human Pain Genetics Database* at well-known stress-related genes including the glucocorticoid receptor (*NR3C1*) and the FK506 binding protein 5 (*FKBP5*).²⁰ Interestingly, the endothelin receptor type B (*EDNRB*), which was previously linked to pain,^{32,34,48} was differentially methylated in both women and men with LBP, making *EDNRB* a potential candidate for further investigation.

In men with LBP, 419 CpGs were found to be differentially methylated, which is 5 times fewer than in women. As in women, DMPs tended towards hypomethylation compared with hypermethylation (57% vs 43%).

In men, we identified a unique LBP DNA methylation signature characterized by significant enrichment for genes from the major histocompatibility complex (MHC) class II, including *HLA-DQA1* and *HLA-DQB2*. Consistent with this, a recent study on the transition from acute to chronic LBP showed significant upregulation of mRNA in blood for antigen presentation pathways (MHC class I and II).¹⁸ MHC class II gene upregulation has been associated with other chronic pain conditions including pancreatitis, postherpetic neuralgia, inguinal hernia repair, complex regional pain syndrome, lumbar disc herniation, rheumatoid arthritis, chronic inflammatory response syndrome, temporomandibular disorders, and LBP. Furthermore, preclinical studies have shown a role for MHC complex in a postherpetic pain model in mice⁴³ and in neuropathic pain in rats,¹⁶ where a contrasting effect was found in female rats.¹⁷

Of the top 10 hypomethylated CpGs in men, 7 mapped to the gene *ZNF718*. Little is known about this protein; zinc finger proteins are involved in transcriptional regulation and it interacts with pain mediators TNF and NGF.⁴⁹ *ZNF718* has been associated with DNA methylation for sex hormone-binding globulin and bioavailable testosterone in males in childhood.² Another study found differential methylation of *ZNF718* in whole blood of severe asthmatics compared with healthy controls.⁵⁰ Further studies are needed to elucidate the role of *ZNF718* in LBP in men.

A major limitation of this study is the small sample size for the discovery cohort, although this is partially mitigated by the inclusion of an independent validation cohort. Despite this limitation, several interesting observations are reported that will guide future, larger scale studies. Increased sample size will allow us to examine the generalisability of these findings and the

interaction of epigenetic reprogramming with risk factors for LBP (eg, early life adversity) and will enable deeper exploration of phenotypic traits (eg, disability severity, anxiety, and depression). Epigenetics may also be important in racial disparities in chronic pain.³

Epigenetic programming is cell type specific, and this study was performed in peripheral T cells. We identified genes with clear function in immune cells (eg, MHC genes and cytokines) that might contribute to chronic pain pathophysiology. Nevertheless, several differentially methylated genes have roles in the central nervous system. It is unclear whether differential methylation in these genes reflects concurrent changes in the central nervous system in humans, but we previously observed an overlap between differentially methylated profiles in T cells and prefrontal cortex in rats with peripheral nerve injury. An important question to be addressed in prospective studies is whether these differentially methylated sites precede or follow the appearance of chronic pain and whether they play a causal role or are a consequence of chronic pain. A related question is whether the epigenetic differences reflect earlier experiences that confer susceptibility and could therefore serve as predictors of risk to developing chronic pain.

In conclusion, chronic LBP is associated with sex-specific epigenome-wide DNA methylation signatures in circulating T cells. We provide proof-of-principle data indicating that the methylation level of a small number of CpGs sites can categorise the pain status (control or LBP) of each participant and that this polygenic methylation score is sex specific. Our study provides preliminary evidence and justification for larger studies to establish associations between chronic LBP, risk factors (eg, previous trauma, smoking), comorbidities (eg, anxiety, depression, and impaired sleep), and DNA methylation. Establishing a causal link between DNA methylation and LBP will open up new windows for the use of therapeutics involving epigenetic reprogramming and development of biomarkers for predicting risk of chronic pain.

Disclosures

Authors S. Gregoire, D. Cheishvili, M. Szyf, and L.S. Stone are coinventors on a provisional patent protecting the methylation signatures described here for possible use as a biomarker for chronic pain. D. Cheishvili and M. Szyf are associated with HKG Epitherapeutics that develops epigenetic biomarkers for clinical use. The remaining authors have no conflicts of interest to declare.

Acknowledgements

The authors thank The Alan Edwards Pain Management Unit for access to facilities and infrastructure; Dr Tim Wideman, Dr Elad Lax, Dr Magali Millecamps, Dr Seunghwan (Peter) Lee, and Dr Mark Ware for valuable input; Ms Nioushah Noushi, Ms Lina Naso, and Ms Seonho Jang for technical support; Ms Abi Thayaparan and Mr Kyle Max for quality control audits; Ms Louise Ulliyatt, Ms Sylvie Toupin, and Ms Linda Ferguson for blood collection and encouragement; and the Montreal Clinical Research Institute (IRCM) and Genome Quebec for access to their core services. This study was supported by the Quebec Pain Research Network, the Alan Edwards Pain Management Unit, a Ronald Melzack Fellowship from the Louise and Alan Edwards Foundation to SG, and Canadian Institutes for Health Research PJT-148636 to MS, MM, and LSS. Finally, the authors thank all participants for their contribution to this study.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PR9/A131>.

Article history:

Received 9 April 2021

Received in revised form 21 July 2021

Accepted 5 August 2021

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