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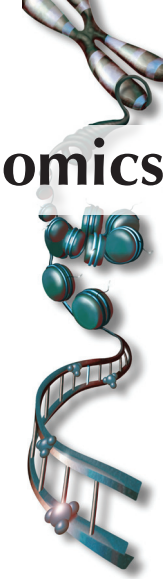
Epigenomics



Variability of response to methadone: genome-wide DNA methylation analysis in two independent cohorts

Aim: Methadone maintenance treatment is characterized by large interindividual dose variability. The aim of this study was to evaluate whether DNA methylations are associated with daily dose of methadone. **Materials & methods**: Subjects stabilized at high (n = 12) or low (n = 12) methadone doses were selected from two independent cohorts (French and Swiss). DNA methylation patterns were analyzed using HumanMethylation450 BeadChips. **Results**: In total, 584 differentially methylated sites were identified in the French cohort corresponding to 352 genes. Of these, 26 were replicated in the Swiss cohort. The methylation status of 13 genes varied similarly in both cohorts and calcium signaling pathway was significantly enriched. **Conclusion**: Our results indicate that differentially methylated sites are associated with methadone daily dose and give insights into the molecular pathways underlying this interindividual dose variability.

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Keywords: addiction • biomarkers • DNA methylation • epigenetics • heroin • methadone

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Maintenance treatments have been shown to reduce the spread of infectious diseases, reduce morbidity and mortality, and improve quality of life [1–4]. Methadone remains the primary replacement drug aimed at abstinence or maintenance worldwide [5].

Treatment retention has become a marker for opioid maintenance treatment success. The retention in opioid maintenance can be relatively low in some programs (30–50% at 6 months) [6,7] with the majority relapsing to illicit opioid use [8]. Using the only biomarker available to follow the compliance of the patients (the concentration of drug metabolites in the urine), it has been shown that retention in methadone maintenance treatment (MMT) is highly dependent on adequate daily dosing [9,10]. The mean daily methadone dose is highly variable across countries, reaching 200 mg/day or more in some countries and ranging between 60 and 100 mg/day in others [11,12]. It has been shown that patients receiving a daily dose of 60–100 mg have better treatment outcomes than those receiving less than 60 mg [13–15]. However, MMT is characterized by a large degree of interindividual variability in daily dose at steady state. Therefore, successful treatment relies in part on individual dose optimization to prevent opiate use, withdrawal and craving. The optimal therapeutic dose at steady state is hard to target or predict before treatment, which may result in a high percentage of patients experiencing withdrawal symptoms and/or persisting in heroin use or dropping out because of relapses [16,17]. Stabilization of patients at steady state may require lengthy trials of dose adjustment, emphasizing the need for biomarkers for individual target daily methadone dose. However, once pharmacological stabilization has occurred, usually in fewer than 3 months, it can take months or years at the same steady state dose to achieve complete remission, and

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at least 2 years of MMT are required before withdrawal should be attempted [18,19].

Genetic variability of several proteins involved in methadone pharmacokinetics and pharmacodynamics has been studied to better understand this variability of dose requirements. Genetic polymorphisms in methadone metabolizing enzymes (mainly CYP2B6) as well as variability in CYP3A activity have been shown to contribute to the observed differences in methadone plasma levels [17,20–22]. Permeability glycoprotein (P-gp) transporter is involved in methadone pharmacokinetics [23,24]; however, the association of its polymorphisms with methadone dose requirement is controversial [17,25–28]. The association of polymorphisms of the mu 1 opioid receptor gene (*OPRM1*) with the required methadone dose has also been studied [28–30]. It has been shown as well that a dopamine D2 receptor polymorphism and polymorphisms in one of its interacting partners, β-Arrestin2, may contribute to the interindividual variability in response to MMT [29,31–33]. Other studies have identified combinatory effects at several loci associated with individual MMT dose requirements [30]. These findings highlight the complexity of factors involved in the daily methadone dose required for effective treatment of opioid addiction. Other factors, mainly environmental, are likely to influence long-term treatment engagement.

Although some genetic polymorphisms participate in the variability of therapeutic response to methadone, we have recently shown that past history of cocaine dependence, current weight, fractioned methadone intake and ethnic background are strongly correlated with methadone maintenance dose [28]. These variables could induce epigenetic modifications contributing to the variability of therapeutic response to MMT. Epigenetic changes caused by drugs of abuse and/or the exposure to long-lasting pharmacological maintenance treatment and the therapeutic consequences of these changes have been poorly explored. It has been found that former heroin addicts stabilized on methadone had targeted differences in DNA methylation at the promoter of *OPRM1* as compared with control subjects [34–36]. These differences in OPRM1 gene methylation could explain the observed decrease in the expression of this receptor [37]. To identify a profile of differentially methylated sites (DMS), in the present study we compare the methylome of patients stable at low and high doses of methadone (HD). First an exploratory analysis was undertaken in a French cohort of MMT patients at steady state. A replication study was performed in an independent cohort of Swiss MMT patients at steady state. The differential methylation signatures of patients in these two cohorts are discussed.

Materials & methods

French cohort

A total of 24 French Caucasian subjects (16 males and 8 females) under steady-state conditions were recruited from the previously described French multicenter study [28]. Steady-state conditions were defined prior to blood collection as stable prescribed daily doses for at least 3 months, no clinical signs of overdose or withdrawal and if a concomitant use of another illicit opiate was present, a standardized clinical interview ruled out criteria for opioid dependence in the past 12 months. Furthermore, subjects with comorbid cocaine, alcohol or benzodiazepine dependence as assessed by DSM-IV criteria at the time of recruitment were not meeting our definition of 'steady state' and thus were not enrolled in this study. Subjects with positive serologic status for HIV were not selected in this cohort. Eleven subjects with positive serologic status for hepatitis C virus (HCV) were included. All patients had four Caucasian grandparents to avoid ethnic stratification bias. Subjects were divided in two groups on the basis of their present and maximal lifetime methadone dose. Written, informed consent was obtained from each subject after they had received a complete description of the study and been given the opportunity to discuss any questions or issues. The study was approved by the Ethics Committee of Paris, Ile-de-France (CPP VI) and was registered to the clinicaltrials.gov website (NCT00894452).

Swiss cohort

A total of 24 Swiss Caucasian subjects (19 males and 5 females) in steady-state conditions were recruited from the previously described Swiss multicenter study [38]. Steady-state conditions were defined as abstinence from heroin and cocaine consumption (based on the self-declaration of the patient and confirmed by the absence of positive tests for opiates or cocaine from urine samples), absence of complaints of withdrawal symptoms and consistent attendance in the therapeutic program all for the 3 months before inclusion in the study and blood collection. Subjects with comorbid cocaine, alcohol or benzodiazepine dependence as assessed by DSM-IV criteria were not included. Three subjects with positive HIV serologic status and eleven subjects with positive serologic status for HCV were included. Subjects were divided in two groups on the basis of their methadone actual and maximal lifetime dose. The study was approved by the local ethics committees of the participating centers. Written, informed consent was obtained from all patients.

Definition of low- & high-dose responders

The French cohort was selected from a multicenter study aiming to identify clinical and genetic param-

eters associated with variability of response to MMT (216 patients included at the time of the beginning of the present study). For the 216 patients, the median methadone dose was 60 mg/day with the 25th percentile at 35 mg/day and the 75th percentile at 80 mg/day. These values were therefore chosen to define the low-dose (LD) group of patients as being <35 mg/kg and high dose (HD) as being >80 mg/kg. Patients selected in the LD group had never received high doses (>80 mg/day). They were also selected for being HIV negative.

The Swiss cohort was also selected from a multicenter study aiming to identify the clinical and genetic parameters associated with the variability of response to MMT, although the daily methadone doses were slightly higher. For the LD group, the 12 selected patients were stabilized at doses <40 mg/day and never received high doses (>105 mg/day). We were not able to reach the same number of patients with the criteria of being HIV negative, and thus this criterion was not applied to the Swiss cohort. Demographic and clinical characteristics of patients included in this study are summarized in Table 1.

DNA extraction & sodium bisulfite conversion

In the French cohort, genomic DNA was extracted from the collected peripheral blood cells with the Wizard Genomic DNA Purification Kit (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions. In the Swiss cohort, Genomic DNA was extracted from EDTA-treated blood samples using the FlexiGene DNA Kit (QIAGEN, Hilden, Germany). For both cohorts, sodium bisulfite conversion of 500 ng of genomic DNA was performed with an EZ DNA Methylation Kit (Zymo Research, CA, USA) according to the manufacturer's instructions.

Illumina infinium HumanMethylation450 BeadChip kit

DNA methylation profiling from 12 patients treated with low and 12 patients treated with high methadone doses was performed in each cohort with HumanMethylation450 BeadChips (Illumina, CA, USA), which simultaneously analyze the methylation status of 485,577 CpG sites covering 99% of all RefSeq genes, with an average of 17 probes per gene, 41% of which are located in proximal promoters. Whole-genome amplification, labeling, hybridization and scanning were performed according to the manufacturer's instruction at Integragen (Evry, France). All arrays were imaged using an Illumina BeadArray Reader. The Infinium platform presents DNA methylation status as a beta value ranging from 0 (no methylation) to 1 (complete methylation). Illumina recommends adding a constant offset α (by default, $\alpha = 100$) to the denominator to regularize Beta value when both methylated and unmethylated probe intensities are low. The beta value (β) is calculated by means of the following equation: $\beta = M/(M + U + \alpha)$ where M = methylated intensity and U = unmethylated intensity. Each locus reports an average beta value obtained from the average of Ms and Us across approximately 30 bead replicates, and individual bead-level measurements are not readily available [39,40]. Raw average βs were exported and analyzed using R (v2.9.2) [41].

Data analysis

All computation and statistical analyses were performed using R. BMIQ (Beta-Mixture Quantile) normalization method and M values computation were used (wateRmelon package) [42]. The data were adjusted for color channel imbalance and background noise. M-values were used for subsequent signal analyses. The Illumina HumanMethylation450 array uses two different chemistries, Infinium I (two probes per CpG locus) and Infinium II (one probe per CpG locus). A β-mixture quantile normalization method was applied to correct this probe design bias [43]. Boxplot of the log2 transformed betas for each sample are presented as supplementary material in Supplementary Figure 1.

Table 1. Clinical data (average ± standard deviation) of the subjects from two cohorts.

Characteristics	French cohort		Swiss cohort	
	LD (n = 12)	HD (n = 12)	LD (n = 12)	HD (n = 12)
Male:female	7:5	9:3	10:2	9:3
Age at sample collection (range)	42.5 ± 10.5 (27–64)	43.2 ± 8.3 (29–56)	36.7 ± 9.2 (25–51)	37.3 ± 7.3 (27–47)
Methadone dose at inclusion (mg/day)	24.7 ± 8.2	109.2 ± 33.1	35.4 ± 8.9	135.8 ± 19.9
Maximum lifetime methadone dose (mg/day)	50.4 ± 17	145.8 ± 63	57.5 ± 15.9	157.5 ± 49.4

HD: High doses of methadone; LD: Low doses of methadone.

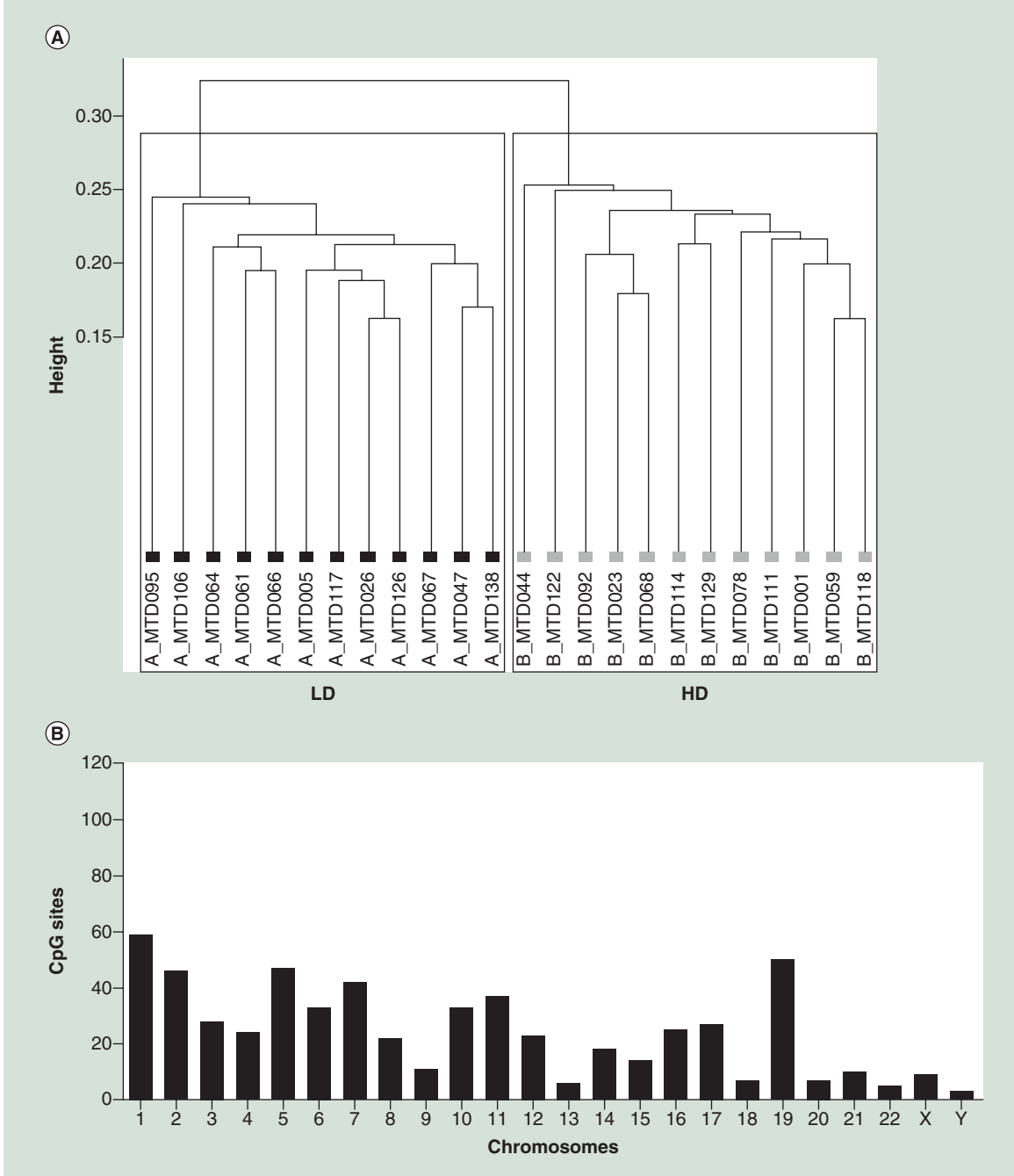

Figure 1. Analysis of differentially methylated sites in the low and high doses of methadone groups of the French cohort. (A) Dendogram plot showing closeness of DNA methylation patterns among the 24 patients from the French cohort. Height is a measure of closeness of either individual data points or clusters. Each branch represents a patient. Cluster analysis discriminated between HD and LD patients (boxes). **(B)** Chromosomal distribution of the 584 differentially methylated sites.
HD: High doses of methadone; LD: Low doses of methadone.

The p-value cut-off for detected probes (different from background measurements) was set at 0.05. M-values distributions of each sample were analyzed using a two-component mixture model to find a cut-off and isolate the unmethylated background CpGs from the significantly methylated CpGs (mixtools package) [44]. We then selected CpGs with a methylation level significantly different from the background for at least half of the samples (P50 list). This method allows discarding of the false positive modulations shown by the very low betas (background). This list is made of 377,060 probes from the 485,577. It has been recently

shown that for medium sample size (i.e., n = 12 per group) Wilcoxon rank–sum test and Student's t-test are both acceptable and produce similar results for DNA methylation microarray analysis [45]. The group comparisons were performed using Student's t-test on BMIQ normalized β values and tests results were associated to the detection scores. The resulting p-values were filtered at 0.05 using the R stats package. In these small series and complex study subjects, correcting for multiple testing could lose potential interest-

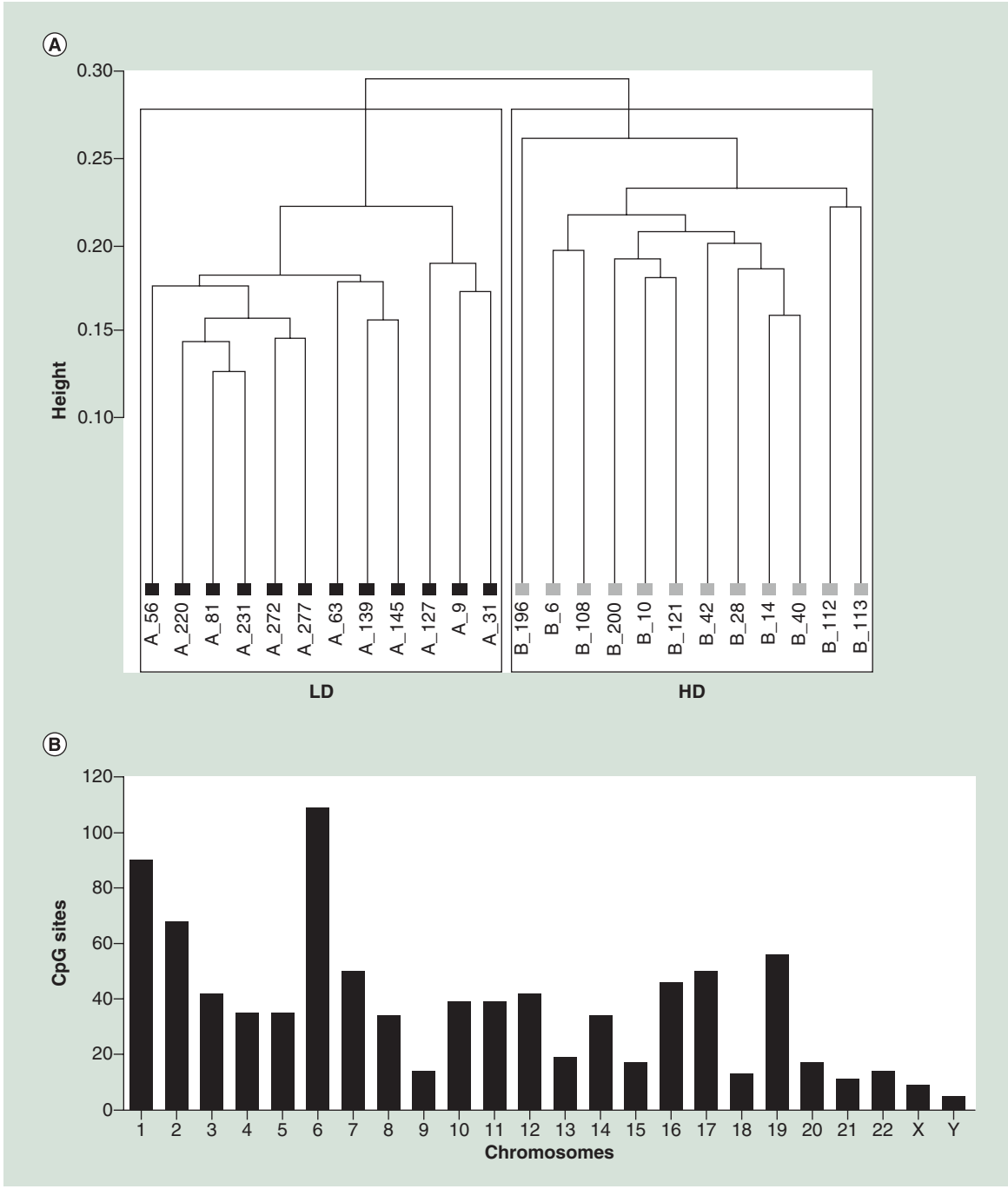

Figure 2. Analysis of differentially methylated sites in the low and high doses of methadone groups of the Swiss cohort. (A) Dendogram plot showing closeness of DNA methylation patterns among the 24 patients from the French cohort. Height is a measure of closeness of either individual data points or clusters. Each branch represents a patient. Cluster analysis discriminated between HD and LD patients (boxes). **(B)** Chromosomal distribution of the 888 differentially methylated sites.
HD: High doses of methadone; LD: Low doses of methadone.

Table 2. The top 20 enriched gene ontology categories in the genes associated with differentially methylated CpG sites in the French cohort.

Categories	Gene count	Raw p-value	Adjusted p-value
Gene expression	72	1.92×10^{-06}	1.61×10^{-02}
Tissue morphology†	32	5.57×10^{-06}	1.61×10^{-02}
Cell-to-cell signaling and interaction†	36	2.16×10^{-05}	1.61×10^{-02}
Cellular assembly and organization	16	2.16×10^{-05}	1.61×10^{-02}
Cellular function and maintenance	33	2.16×10^{-05}	1.61×10^{-02}
Nervous system development and function†	40	2.16×10^{-05}	1.61×10^{-02}
Tissue development†	74	2.16×10^{-05}	1.61×10^{-02}
Digestive system development and function	32	2.47×10^{-05}	1.61×10^{-02}
Embryonic development†	61	2.47×10^{-05}	1.61×10^{-02}
Organ development†	48	2.47×10^{-05}	1.61×10^{-02}
Organismal development†	77	2.47×10^{-05}	1.61×10^{-02}
Connective tissue development and function†	39	6.18×10^{-05}	1.61×10^{-02}
Organ morphology†	55	6.18×10^{-05}	1.61×10^{-02}
Skeletal and muscular system development and function†	28	6.18×10^{-05}	1.61×10^{-02}
Organismal survival	75	9.13×10^{-05}	1.61×10^{-02}
Cell death and survival†	103	1.11×10^{-04}	1.61×10^{-02}
Cancer†	211	1.89×10^{-04}	1.61×10^{-02}
Gastrointestinal disease	113	2.44×10^{-04}	1.61×10^{-02}
Organismal injury and abnormalities†	42	2.65×10^{-04}	1.61×10^{-02}
Reproductive system disease	33	2.65×10^{-04}	1.61×10^{-02}

Ranks were determined by adjusted p-value (Benjamini–Hochberg multiple testing correction).
†Found both in the French and Swiss cohorts.

ing findings; we therefore chose to replicate the study in an independent cohort as a matter of controlling findings of chance. Volcano plots of -log10 (p-value) against log2 (ratio) obtained for the two cohorts are presented in Supplementary Figure 2. In order to statistically select the significant modulations we used a computed threshold on amplitude (absolute value of means difference) based on the CpG rejected from the P50 list considered as unmethylated or background values (with less than 50% of M-values characterized as methylated) to compute their modulation amplitudes. However the arbitrary 1.2-fold threshold was more stringent than the computed one and was eventually chosen to consider a limited number of CpG in order to facilitate interpretation. Probe lists were then filtered by 1.2-fold-change variation of the methylation status. A clustering step was performed after supervised analysis using hierarchical clustering in R (stats package) [41] using the Spearman correlation similarity measure and the average linkage algorithm to test the generated list by classifying the samples in expected groups.

Pathway analysis

Data were subsequently analyzed using the Web-based Gene Set Analysis Toolkit (WebGestalt [46]) to identify enriched GO (gene ontology) categories in the genes associated with differentially methylated CpG sites in their regulatory regions in the two cohorts. The enrichment of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways among genes that exhibit differential DNA methylation in the HD and LD groups of the two cohorts was also tested. Enrichment for specific pathways and biological functions was determined relative to the proprietary database, after adjustment for multiple testing using the Benjamini–Hochberg method the criterion for significance was $p < 0.05$.

Results

Quality control & clustering

In the French cohort, genome-wide DNA methylation profiling of genomic DNA samples from 12 LD and 12 HD MMT patients resulted in a raw data matrix of 358,537 probes after stringent quality control analysis (see section 'Materials & methods'). This raw data matrix was then subjected to an unsupervised clustering analysis. No aberrant samples were found, allowing the subsequent analysis of all patients (Supplementary Figure 3). A total of 584 CpG sites had significantly different DNA methylation levels between the HD and LD groups. Among these, 291 showed hypermethylation (>1.2-fold) in HD patients, and 293 showed hypomethylation (<0.8-fold). Cluster analysis of the 584 CpG sites successfully discriminated between HD and LD patients (Figure 1A). We determined the chromosomal distribution of differentially methylated CpG sites in HD and LD patients (Figure 1B), and detailed analysis revealed no statistically overrepresented chromosomes compared with the chromosomal distribution of all analyzed CpG sites on the Infinium HumanMethylation450 BeadChip (see Supplementary Table 1 for Chi squared analyses).

In the Swiss cohort, as previously described, raw data obtained from 12 LD and 12 HD MMT patients were subjected to quality control analysis, which resulted in a raw data matrix of 358,377 probes. After unsupervised clustering analysis, no aberrant samples were found allowing for the subsequent analysis of the 24 patients (Supplementary Figure 3). Significantly different DNA methylation levels between the HD and LD groups were found at 888 CpG sites, with 382 hypermethylated (>1.2-fold) and 506 hypomethylated (<0.8-fold) CpG sites in patients from the HD group. Cluster analysis of the 888 CpG sites successfully discriminated between the HD and LD patients (Figure 2A). Analysis of the chromosomal

Table 3. The top 20 enriched gene ontology categories in the genes associated with differentially methylated CpG sites in the Swiss cohort.

Categories	Gene count	Raw p-value	Adjusted p-value
Inflammatory disease	69	1.46×10^{-07}	9.48×10^{-03}
Neurological disease	109	1.46×10^{-07}	1.45×10^{-02}
Skeletal and muscular disorders	109	1.46×10^{-07}	1.53×10^{-02}
Cellular growth and proliferation†	173	2×10^{-07}	1.31×10^{-02}
Connective tissue development and function†	48	4.95×10^{-07}	1.45×10^{-02}
Embryonic development†	89	4.95×10^{-07}	1.45×10^{-02}
Nervous system development and function†	116	4.95×10^{-07}	1.46×10^{-02}
Organ development†	78	4.95×10^{-07}	1.45×10^{-02}
Organ morphology†	86	4.95×10^{-07}	1.45×10^{-02}
Organismal development†	134	4.95×10^{-07}	1.45×10^{-02}
Skeletal and muscular system development and function†	65	4.95×10^{-07}	1.45×10^{-02}
Tissue development†	77	4.95×10^{-07}	1.45×10^{-02}
Tissue morphology†	123	6.67×10^{-07}	1.46×10^{-02}
Cancer†	311	2.89×10^{-06}	1.45×10^{-02}
Cell death and survival†	166	3.21×10^{-06}	1.4×10^{-02}
Cellular development	161	7.16×10^{-06}	1.31×10^{-02}
Metabolic disease	80	2.39×10^{-05}	1.43×10^{-02}
Behavior	57	2.6×10^{-05}	1.28×10^{-02}
Hematological system development and function	93	2.66×10^{-05}	1.33×10^{-02}
Psychological disorders	69	7.58×10^{-05}	1.4×10^{-02}

Ranks were determined by adjusted p-value (Benjamini–Hochberg multiple testing correction).
†Found both in the French and Swiss cohorts.

distribution of the differentially methylated CpG sites in HD and LD patients (Figure 2B) showed that DMS are evenly distributed to all chromosomes (see Supplementary Table 1 for Chi squared analyses).

Differentially methylated CpG sites & genes in subjects from the HD & LD groups

As shown in Supplementary Table 2 differentially methylated CpG islands were found to be distributed mostly within exons: 77% and 73% of the DMS in the French and Swiss cohorts, respectively. The DMS present in promoter regulatory regions (500 bps upstream and 2000 bps downstream of the transcription start site) represent 9% and 13.5% in the French and Swiss cohort, respectively. The Infinium HumanMethylation450 BeadChip is characterized by the presence of multiple probes per gene; different CpG sites in the two cohorts may therefore target the same gene. Thus, we next analyzed the genes potentially regulated by the CpG sites differentially methylated between the HD and LD groups (Table 2). Among the regulatory CpG sites, 70% were present in genes that were annotated in the databases (405 sites) in the French cohort. The complete list of the genes displaying differentially methylated CpG sites in the French cohort is presented in Supplementary Table 3. The subsequent analyses were undertaken on these 405 sites corresponding to 352 unique genes present in the databases. Among them, 100 (34.7%) displayed a hypomethylated profile in HD patients compared with LD patients, and 188 (65.3%) displayed a hypermethylated profile. Similar proportions were found in the Swiss cohort with a total of 638 CpG sites (72%) corresponding to 572 unique annotated genes in the databases. See Supplementary Table 4 for a detailed list of the genes displaying differentially methylated CpG sites in the HD group compared with the LD group in the Swiss cohort. Among these genes, 151 (38%) were found to be hypomethylated, and 245 (62%) were hypermethylated in the HD group (Table 2).

Over-represented categories in each cohort

We used WebGestalt to identify the most relevant GO (gene ontology) categories and pathways associated with high daily methadone doses compared with low daily doses. As seen in Table 2, the top 20 overrepresented GO categories in the French cohort were related to diseases such as cancer, gastrointestinal disease and reproductive system disease but also include metabolic processes (gene expression); cellular processes (cell–cell signaling, cellular assembly, cellular maintenance); and developmental processes (tissue development, nervous system development, embryonic development, connective tissue development, organ development, organismal development, tissue morphology and organ morphology). In the Swiss cohort, diseases such as inflammatory disease, cancer, neurological disease, skeletal and muscular disorders, metabolic disease and psychological disorders were overrepresented (Table 3). Categories present in both cohorts are indicated; they include several categories

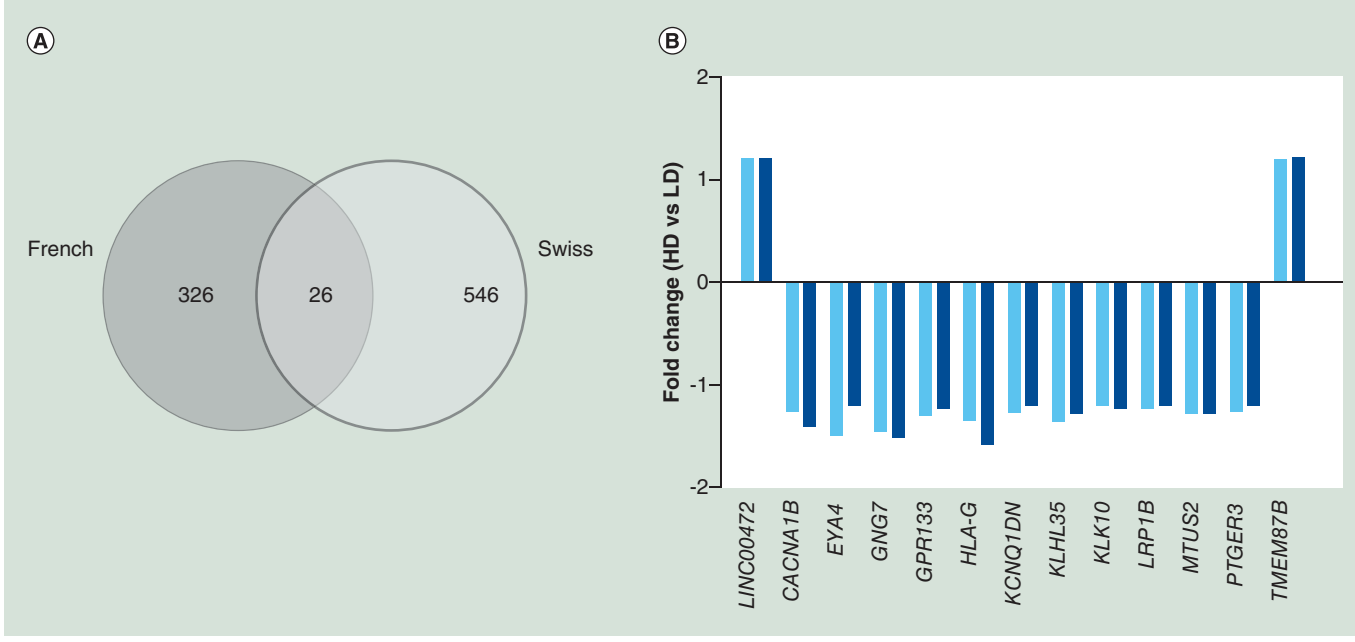

Figure 3. Analysis of the genes associated with differentially methylated sites in the two populations. (A) Venn diagram of the genes associated with differentially methylated CpG sites in the HD and LD groups in the French and Swiss cohorts. **(B)** Fold change of methylation status of the common genes in the French and Swiss cohorts.
HD: High doses of methadone; LD: Low doses of methadone.

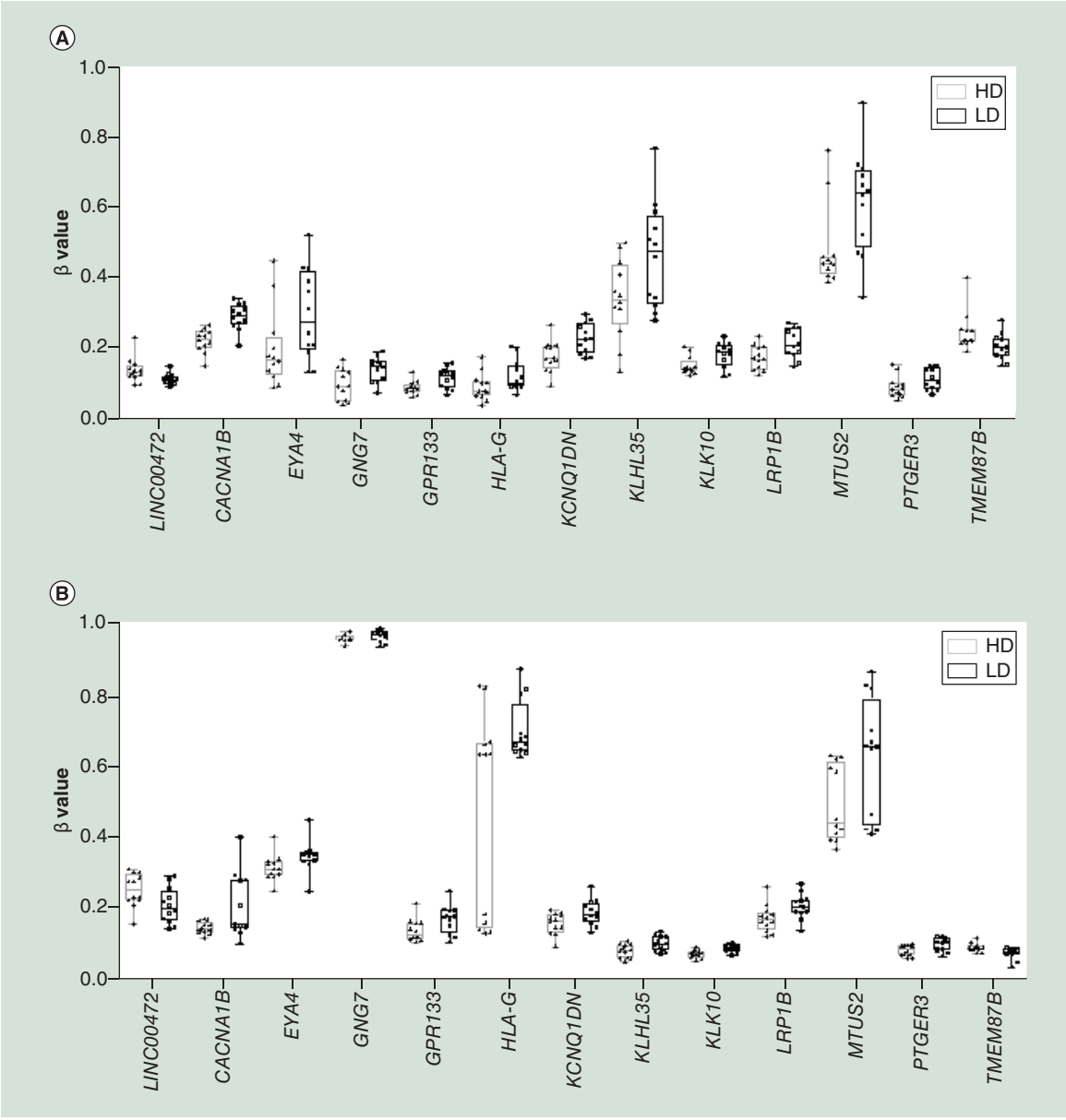

Figure 4. Distribution of methylation status. The 13 genes that vary similarly in the **(A)** French and **(B)** Swiss cohorts are shown. The bottom and the top of the boxes represent the 25th and 75th percentiles, respectively. The median is represented by horizontal lines. Individual values in the HD and LD groups are superimposed.
HD: High doses of methadone; LD: Low doses of methadone.

related to developmental processes (tissue development; connective tissue development; embryonic development; nervous system development; organ development; organismal development; skeletal and muscular organ development; tissue morphology; and organ morphology); cellular processes (cell growth and proliferation, cell death and survival) and a single disease (cancer).

Genes associated with differentially methylated CpG sites in both cohorts

As shown in Figure 3A, 26 genes were found in common between the two cohorts and among these 13 displayed a similar methylation status in the HD group compared with the LD group in both cohorts. Two genes (*LINC00472* and *TMEM87B*) were hypermethylated, whereas 11 genes (*CACNA1B*, *EYA4*, *GNG7*, *GPR133*, *HLA-G*, *KCNQ1DN*, *KLHL35*, *KLK10*, *LRP1B*, *MTUS2*, *PTGER3*) were hypomethylated in the HD group in both tested cohorts (Figure 3B). The individual distribution of β values for these 13 genes in the HD and LD groups of the two cohorts are shown in Figure 4. Analysis of the DMS of these 13 genes showed that the number of sites affected is not always the same in the two cohorts (Table 4). Furthermore,

Table 4. Differentially methylated CpG sites of the thirteen genes found in common in the two populations.

Gene	French					Swiss				
	Illumina probe IDs	Position	Average β values		p-value	Illumina probe IDs	Position	Average β values		p-value
			HD	LD				HD	LD	
LINC00472	cg24772267	72187463	0.138	0.114	0.037	cg15832847	72187368	0.246	0.02	0.04
CACNA1B	cg13811936 cg14022794	140136822 140137278	0.15 0.289	0.207 0.365	0.002 0.0003	cg11827453	140037234	0.014	0.199	0.042
EYA4	cg17985029	133603340	0.196	0.292	0.039	cg11942956 cg25075347	133773494 133604163	0.145 0.469	0.116 0.566	0.024 0.005
GNG7	cg22323942	2494655	0.097	0.141	0.014	cg24299136	2462707	0.630	0.653	0.028
GPR133	cg02717046	130004741	0.089	0.116	0.014	cg24301724	130182997	0.134	0.166	0.041
HLA-G	cg03149560	29904358	0.091	0.122	0.045	cg06316104 cg25046571	29904964 29902636	0.563 0.359	0.814 0.568	0.011 0.012
KCNQ1DN	cg03947814 cg04457979 cg07592963 cg22389375	2846995 2847223 2847225 2847246	0.22 0.108 0.109 0.267	0. 284 0.174 0.136 0.339	0.003 0.03 0.045 0.02	cg14582642	2846895	0.153	0.184	0.034
KLHL35	cg05353869	74817192	0.334	0.455	0.037	cg03934926	74819483	0.077	0.099	0.015
KLK10	cg09226684	56211905	0.15	0.180	0.028	cg15910208	56214400	0.07	0.086	0.002
LRP1B	cg22830273	142605753	0.172	0.211	0.017	cg22830273	142605753	0.167	0.201	0.027
MTUS2	cg05122437	28953092	0.468	0.601	0.017	cg05122437	28953092	0.485	0.62	0.033
PTGER3	cg06937997	71284688	0. 91	0.115	0.048	cg23647191	71286082	0.079	0.096	0.031
TMEM87B	cg02414648	112529227	0.242	0.202	0.027	cg04439376	112528601	0.087	0.071	0.02

Chromosomal positions are based on hg18 (build 36). The CpG sites in bold are the same in the French and Swiss cohorts.
HD: High doses of methadone; LD: Low doses of methadone.

the DMS are different in the two populations except for *LRP1B* and *MTUS2* for which the same site is differentially methylated in the HD patients as compared with the LD in the two populations. We next performed a KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis to identify biological pathways enriched for genes that exhibit differential DNA methylation in HD compared with LD methadone stabilized patients. The 26 common genes were analyzed, and the human genome was used as background. Only the calcium signaling pathway, with two genes (*CACNA1B* and *PTGER3*), was found to be significantly enriched in the HD group (Table 5).

Discussion

In this study, we compared the genome-wide DNA methylation profiles of patients stabilized at high daily methadone doses to the profiles of patients at steady state with low daily doses in cohorts from two different countries (France and Switzerland). Differential methylation patterns obtained here are consistent between the two cohorts studied. We identified a distinct set of epigenetic signatures in subjects stabilized at HD in the two cohorts. The clinical use of methadone differs between these countries however several overrepresented cellular functions were identified, from the differentially methylated genes, in both cohorts. We also replicated in the two independent cohorts a set of 13 differentially methylated genes in the HD and LD patients.

The current study provides a basic understanding of DNA methylation in peripheral blood cells of former heroin addicts stabilized by MMT with a high or a low daily methadone dose. To our knowledge, this study is the first to analyze genome-wide DNA methylation differences between HD and LD MMT stable patients in two independent cohorts. We observed different CpG methylation in HD and LD groups of patients in two independent cohorts of stable patients. The signatures obtained from these cohorts displayed similar proportions of hypermethylated and hypomethylated CpG sites in the HD groups compared with the LD groups. In the samples from this study, we were unable to replicate the OPRM1 gene hypermethylation in former addicts on MMT that was reported previously [35]. This is most likely because the previous study compared patients stabilized in methadone treatment to control subjects, whereas our study focused on epigenetic differences between former addicts according to their stabilizing methadone dose in MMT.

Enrichment studies found that the genes associated with the differentially methylated CpG signature belong to biological processes such as development, morphology, cell signaling and cell death. These processes could be important to mediate methadone-induced signaling in cells. Among the candidate genes whose expression pattern could be affected by these two signatures, 13 displayed similar modulations of their methylation status in the French and Swiss cohorts. None of these genes have been previously associated with heroin or other substance addiction, response to methadone or response to other drugs. They therefore represent new potential avenues of research in the field of variability of response to daily methadone dose. It is interesting to note that the calcium signaling pathway, known to be elicited by Mu receptor agonists such as methadone, was found to be significantly enriched in the HD group.

Modification of DNA methylation at CpG sites in the vicinity of these 13 genes could be the result of several mechanisms. One mechanism could be an innate predisposing factor for vulnerability to be stabilized with HD or LD of methadone. Another mechanism could be acquired as the result of life events which occurred before heroin was first taken. It has been shown that attention deficit hyperactivity disorder (ADHD), history of trauma, sexual abuse and conduct disorders are highly prevalent in patients entering MMT [47–49]. These factors have been shown to correlate with DNA methylation modifications, which may be associated with susceptibility to or a consequence of drug abuse, in peripheral blood cells [50–53]. Acquired changes in DNA methylation could also result from chronic heroin use. It has been shown that the daily methadone dose required to stabilize patients could be correlated to the severity of addiction [54]. Consumption of high doses of heroin, length of heroin use, repeated cycles of heroin use and withdrawal are all factors that might modulate DNA methylation and predispose the patients to be stabilized with HD. A significant relationship was found in the French cohort between an indirect marker of addiction severity and daily methadone dose [28]. Preliminary results suggest other association with previously identified clinical factors such as past history of cocaine dependence, weight and fractioned methadone intake in the French sample used in this study. Further studies assessing the association between those clinical factors and the methylation status observed here are needed. The objective could be to describe a multifactorial methylation model based on several correlated clinical variables.

The observed differential DNA methylation between HD and LD groups could also result from methadone treatment itself. Patients in the two cohorts enrolled in this study had a median history of 3 to 4 years of MMT. This long-term daily consumption of methadone could induce cumulative dose-dependent modifications of DNA methylation status. It has been suggested that chronic opioid use could induce increased DNA methylation at a global methylation site (LINE1) and at the promoter of *OPRM1* in blood cells of chronic pain patients treated with opioids compared with control subjects [34]. Again, these loci were not found to be differentially methylated in our study, which has a different design, as all of the patients included here were former heroin users. Longitudinal studies of comparative genome-wide DNA methylation profiles of heroin addicts at entrance into MMT and at steady-state should allow for the determination of the innate and acquired causes of observed differential DNA methylation for these 13 genes.

This study has some limitations. First, the strong selection criteria applied to define both HD and LD groups limited the sample size, particularly for the LD group. Therefore the two groups could not be adjusted for severity of their previous heroin addiction. However, these data were available in the French cohort and the LD and HD groups were not different on this dimension as assessed by the available variables (age of first heroin use, intravenous route for heroin, days of heroin use per week, duration of methadone treatment and persistent occasional heroin use during the past 12 months of methadone treatment). Although our sample size is relatively small for detecting small

Table 5. Kyoto Encyclopedia of Genes and Genomes pathways with enrichment of genes that exhibit differential DNA methylation in patients from the high doses of methadone group compared to the low doses of methadone group in both cohorts studied.

Pathway (number of genes in pathway)	Observed number of genes	Expected number of genes	Ratio observed/ expected	Raw p-value	Adjusted p-value	Observed genes
Calcium signaling pathway (177)	2	0.1	19.49	0.0047	0.0094	*PTGER3*, *CACNA1B*

p-values have been adjusted for multiple testing using the Benjamini–Hochberg method.
HD: High doses of methadone; LD: Low doses of methadone.

effects in a genome-wide analysis, it is noteworthy that the analyzed CpG sites reached levels of significance. Moreover, the sample size is comparable to similar studies [55,56]. Second, the genome-wide data generated in this study are from genomic DNA of blood cells, which limits inference of the effects of methadone on the brain. It has been found in control subjects that DNA methylation at promoter CpG islands is strongly conserved across brain areas and blood suggesting that peripheral tissues may be useful in epigenetic studies of complex neurobiological phenotypes [57]. However, additional studies are necessary to determine if the modulations induced by drugs such as methadone can be similar in different tissues. A third limitation of this study is that differences in methylation levels could not be verified by pyrosequencing or be extrapolated directly to modulations in gene expression levels and disruption of pathways. These cohorts of former heroin addicts stabilized with a maintenance drug are very difficult to implement. The collected DNAs have been used in several ongoing genotyping projects [20,28,31–32,38,58–65 and VORSPAN ET AL., UNPUBLISHED RESULTS] and we could not perform pyrosequencing due to limited amount of DNA left for some of the patients in the LD group. Nine percent and 13.5% of the differentially methylated CpG sites in the French and Swiss cohorts, respectively, are located within known regulatory gene regions. RNA samples from blood cells of patients treated with a stable dose of methadone were not collected in the two studied cohorts precluding the analysis of gene expression in parallel with the DNA methylation analysis. Studies in other cohorts, selecting homogeneous group of patients based on their chronic infectious diseases status will be required to extrapolate these observations to other groups of patients.

Conclusion & future perspective

Despite these limitations, our study is the first to use a genome-wide analysis to identify DNA methylation differences associated with high or low daily dosages in peripheral DNA samples from MMT-stabilized patients. It is not possible from our results to differentiate if the observed differences in DNA methylation characterize a predisposition to high or low methadone daily doses or if they result from methadone pharmacotherapy at high or low doses. A study of DNA methylation patterns in peripheral blood cells at the initiation of MMT and at steady state should allow differentiation between these two possibilities. Long-term retention in an MMT program and variability of daily methadone doses at steady state are influenced by factors that have been linked to differences in DNA methylation patterns, such as environmental and sociodemographic factors. Therefore, DNA methylation could be used as a surrogate biomarker for the therapeutic response to methadone.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/epi.15.110

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Executive summary

- Methadone maintenance treatment is characterized by a large degree of interindividual dose variability among patients.
- The aim of this study was to evaluate whether differences in DNA methylations are associated with daily dose of methadone.
- Caucasian subjects stabilized at high and low methadone daily doses were selected from two independent samples (French and Swiss) to conduct replication studies.
- Genome-wide DNA methylation was examined in peripheral blood cells of 12 patients stabilized at a low dose (LD) and 12 patients stabilized at a high dose (HD) in each cohort.
- A total of 584 and 888 differentially methylated sites, associated with the doses of methadone, were identified in the French and Swiss cohorts, respectively.
- We identified a set of 13 genes whose methylation status varied similarly in both cohorts.
- Among these, the calcium signaling pathway was found significantly enriched
- This work is the first to identify DNA methylation differences associated with daily methadone dose.
- The findings of this study are from a cross-sectional study so causal conclusions cannot be drawn. Future prospective studies should allow to differentiate if the observed differences characterize a predisposition to methadone daily HD or LD, or if they result from methadone pharmacotherapy at HD or LD.

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References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Gibson A, Degenhardt L, Mattick RP, Ali R, White J, O'Brien S. Exposure to opioid maintenance treatment reduces long-term mortality. *Addiction* 103(3), 502–468 (2008).

2 Mattick RP, Breen C, Kimber J, Davoli M. Methadone maintenance therapy versus no opioid replacement therapy for opioid dependence. *Cochrane Database Syst. Rev.* (3), CD002209 (2009).

3 Fucci N, De Giovanni N. Methadone in hair and sweat from patients in long-term maintenance therapy. *Ther. Drug Monitor.* 29(4), 452–454 (2007).

4 Dennis BB, Naji L, Bawor M *et al.* The effectiveness of opioid substitution treatments for patients with opioid dependence: a systematic review and multiple treatment comparison protocol. *Syst. Rev.* 3, 105 (2014).

• **This is a recent systematic review comparing the effects of all available maintenance therapies on the treatment of opioid dependence.**

5 Somogyi AA, Barratt DT, Ali RL, Coller JK. Pharmacogenomics of methadone maintenance treatment. *Pharmacogenomics* 15(7), 1007–1027 (2014).

6 Bell J, Mutch C. Treatment retention in adolescent patients treated with methadone or buprenorphine for opioid dependence: a file review. *Drug Alcohol Rev.* 25(2), 167–171 (2006).

7 Mattick RP, Kimber J, Breen C, Davoli M. Buprenorphine maintenance versus placebo or methadone maintenance for opioid dependence. *Cochrane Database Syst. Rev.* (2), CD002207 (2008).

8 Winstock AR, Lintzeris N, Lea T. "Should I stay or should I go?" Coming off methadone and buprenorphine treatment. *Int. J. Drug Policy* 22(1), 77–81 (2010).

9 Epstein DH, Schmittner J, Umbricht A, Schroeder JR, Moolchan ET, Preston KL. Promoting abstinence from cocaine and heroin with a methadone dose increase and a novel contingency. *Drug Alcohol Depend.* 101(1–2), 92–100 (2009).

10 Farre M, Mas A, Torrens M, Moreno V, Cami J. Retention rate and illicit opioid use during methadone maintenance interventions: a meta-analysis. *Drug Alcohol Depend.* 65(3), 283–290 (2002).

11 Kreek MJ, Borg L, Ducat E, Ray B. Pharmacotherapy in the treatment of addiction: methadone. *J. Addict. Dis.* 29(2), 200–216 (2010).

12 Mullen L, Barry J, Long J *et al.* A national study of the retention of irish opiate users in methadone substitution treatment. *Am. J. Drug Alcohol Abuse* 38(6), 551–558 (2012).

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

13 Faggiano F, Vigna-Taglianti F, Versino E, Lemma P. Methadone maintenance at different dosages for opioid dependence. *Cochrane Database Syst. Rev.* (3), CD002208 (2003).

14 Strain EC, Bigelow GE, Liebson IA, Stitzer ML. Moderate- vs high-dose methadone in the treatment of opioid dependence: A randomized trial. *J. Am. Med. Assoc.* 281(11), 1000–1005 (1999).

15 Fareed A, Casarella J, Roberts M *et al.* High dose versus moderate dose methadone maintenance: Is there a better outcome? *J. Addict. Dis.* 28(4), 399–405 (2009).

16 Hanna J, Foster DJ, Salter A, Somogyi AA, White JM, Bochner F. Within- and between- subject variability in methadone pharmacokinetics and pharmacodynamics in methadone maintenance subjects. *Br. J. Clin. Pharmacol.* 60(4), 404–413 (2005).

17 Fonseca F, De La Torre R, Diaz L *et al.* Contribution of cytochrome P450 and ABCB1 genetic variability on methadone pharmacokinetics, dose requirements, and response. *PLoS ONE* 6(5), e19527 (2011).

18 Moolchan ET, Hoffman JA. Phases of treatment: a practical approach to methadone maintenance treatment. *Int. J. Addict.* 29(2), 135–160 (1994).

19 Kleber HD. Methadone maintenance 4 decades later: Thousands of lives saved but still controversial. *J. Am. Med. Assoc.* 300(19), 2303–2305 (2008).

20 Crettol S, Deglon JJ, Besson J *et al.* ABCB1 and cytochrome P450 genotypes and phenotypes: influence on methadone plasma levels and response to treatment. *Clin. Pharmacol. Ther.* 80(6), 668–681 (2006).

21 Perez De Los Cobos J, Sinol N, Trujols J *et al.* Association of CYP2D6 ultrarapid metabolizer genotype with deficient patient satisfaction regarding methadone maintenance treatment. *Drug Alcohol Depend.* 89(2–3), 190–194 (2007).

22 Wang SC, Ho IK, Tsou HH *et al.* CYP2B6 polymorphisms influence the plasma concentration and clearance of the methadone S-enantiomer. *J. Clin. Psychopharmacol.* 31(4), 463–469 (2011).

23 Wang JS, Ruan Y, Taylor RM, Donovan JL, Markowitz JS, Devane CL. Brain penetration of methadone (R)- and (S)-enantiomers is greatly increased by P-glycoprotein deficiency in the blood-brain barrier of Abcb1a gene knockout mice. *Psychopharmacology (Berl.)* 173(1–2), 132–138 (2004).

24 Tournier N, Chevillard L, Megarbane B, Pirnay S, Scherrmann JM, Decleves X. Interaction of drugs of abuse and maintenance treatments with human P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2). *Int. J. Neuropsychopharmacol.* 13(7), 905–915 (2010).

25 Coller JK, Barratt DT, Dahlen K, Loennechen MH, Somogyi AA. ABCB1 genetic variability and methadone dosage requirements in opioid-dependent individuals. *Clin. Pharmacol. Ther.* 80(6), 682–690 (2006).

26 Levran O, O'Hara K, Peles E *et al.* ABCB1 (MDR1) genetic variants are associated with methadone doses required for effective treatment of heroin dependence. *Hum. Mol. Genet.* 17(14), 2219–2227 (2008).

27 Dennis BB, Bawor M, Thabane L, Sohani Z, Samaan Z. Impact of ABCB1 and CYP2B6 genetic polymorphisms on methadone metabolism, dose and treatment response in patients with opioid addiction: A systematic review and meta-analysis. *PLoS ONE* 9(1), e86114 (2014).

28 Mouly S, Bloch V, Peoc'h K *et al.* Methadone dose in heroin-dependent patients: role of clinical factors, comedications, genetic polymorphisms and enzyme activity. *Brit. J. Clin. Pharmacol.* 79(6), 967–977 (2015).

• **Study reporting that past history of cocaine dependence, current weight, fractioned methadone intake and ethnic background are better predictors of methadone maintenance dose than genetic polymorphisms.**

29 Levran O, Peles E, Randesi M *et al.* Association of genetic variation in pharmacodynamic factors with methadone dose required for effective treatment of opioid addiction. *Pharmacogenomics* 14(7), 755–768 (2013).

30 Barratt DT, Coller JK, Hallinan R *et al.* ABCB1 haplotype and OPRM1 118A > G genotype interaction in methadone maintenance treatment pharmacogenetics. *Pharmacogenomics Pers. Med.* 5, 53–62 (2012).

31 Crettol S, Besson J, Croquette-Krokar M *et al.* Association of dopamine and opioid receptor genetic polymorphisms with response to methadone maintenance treatment. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32(7), 1722–1727 (2008).

32 Oneda B, Crettol S, Bochud M *et al.* Beta-arrestin2 influences the response to methadone in opioid-dependent patients. *Pharmacogenomics J.* 11(4), 258–266 (2011).

33 Barratt DT, Coller JK, Somogyi AA. Association between the DRD2 A1 allele and response to methadone and buprenorphine maintenance treatments. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 141B(4), 323–331 (2006).

34 Doehring A, Oertel BG, Sittl R, Lotsch J. Chronic opioid use is associated with increased DNA methylation correlating with increased clinical pain. *Pain* 154(1), 15–23 (2013).

•• **Reports increased DNA methylation of the *OPRM1* gene and LINE-1 element in a cohort of methadone maintained as compared with controls and a cohort of opioid-treated as compared with nontreated patients.**

35 Nielsen DA, Yuferov V, Hamon S *et al.* Increased OPRM1 DNA methylation in lymphocytes of methadone-maintained former heroin addicts. *Neuropsychopharmacol.* 34(4), 867–873 (2009).

•• **Reports differential DNA methylation of the *OPRM1* gene in methadone maintained subjects as compared with controls.**

36 Nielsen DA, Hamon S, Yuferov V *et al.* Ethnic diversity of DNA methylation in the oprm1 promoter region in lymphocytes of heroin addicts. *Hum. Genet.* 127(6), 639–649 (2010).

•• **Shows the ethnic specificity of CpG sites and differences in DNA methylation at the *OPRM1* gene in former heroin addicts as compared with controls.**

37 Toskulkao T, Pornchai R, Akkarapatumwong V, Vatanatunyakum S, Govitrapong P. Alteration of lymphocyte opioid receptors in methadone maintenance subjects. *Neurochem. Int.* 56(2), 285–290 (2010).

38 Crettol S, Deglon JJ, Besson J *et al.* Methadone enantiomer plasma levels, CYP2B6, CYP2C19, and CYP2C9 genotypes, and response to treatment. *Clin. Pharmacol. Ther.* 78(6), 593–604 (2005).

39 Dunning MJ, Barbosa-Morais NL, Lynch AG, Tavare S, Ritchie ME. Statistical issues in the analysis of illumina data. *BMC Bioinformatics* 9, 85 (2008).

40 Wong WC, Loh M, Eisenhaber F. On the necessity of different statistical treatment for illumina beadchip and affymetrix genechip data and its significance for biological interpretation. *Biol. Direct* 3, 23 (2008).

41 R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria ISBN 3-900051-07-0. www.R-project.org

42 Pidsley R, Cc YW, Volta M, Lunnon K, Mill J, Schalkwyk LC. A data-driven approach to preprocessing illumina 450k methylation array data. *BMC Genomics* 14, 293 (2013)

43 Teschendorff AE, Marabita F, Lechner M *et al.* A beta-mixture quantile normalization method for correcting probe design bias in illumina infinium 450 k DNA methylation data. *Bioinformatics* 29(2), 189–196 (2013).

44 Benaglia T, Chauveau D, Hunter DR, Young DS. Mixtools: an R package for analyzing mixture models. *J. Stat. Softw.* 32(6), 29 (2009).

45 Li D, Xie Z, Pape ML, Dye T. An evaluation of statistical methods for DNA methylation microarray data analysis. *BMC Bioinformatics* 16, 217 (2015).

46 WEB-based GEne SeT AnaLysis Toolkit. http://bioinfo.vanderbilt.edu

47 King VL, Brooner RK, Kidorf MS, Stoller KB, Mirsky AF. Attention deficit hyperactivity disorder and treatment outcome in opioid abusers entering treatment. *J. Nerv. Ment. Dis.* 187(8), 487–495 (1999).

48 Carpentier PJ, Van Gogh MT, Knapen LJ, Buitelaar JK, De Jong CA. Influence of attention deficit hyperactivity disorder and conduct disorder on opioid dependence severity and psychiatric comorbidity in chronic methadone-maintained patients. *Eur. Addict. Res.* 17(1), 10–20 (2011).

49 Peles E, Potik D, Schreiber S, Bloch M, Adelson M. Psychiatric comorbidity of patients on methadone maintenance treatment with a history of sexual abuse. *Eur. Neuropsychopharmacol.* 22(12), 883–891 (2012).

50 Mehta D, Klengel T, Conneely KN *et al.* Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder. *Proc. Natl Acad. Sci. USA* 110(20), 8302–8307 (2013).

51 Perroud N, Paoloni-Giacobino A, Prada P *et al.* Increased methylation of glucocorticoid receptor gene (*NR3C1*) in adults with a history of childhood maltreatment: A link with the severity and type of trauma. *Transl. Psychiatr.* 1, e59 (2011).

52 Van Mil NH, Steegers-Theunissen RP, Bouwland-Both MI *et al.* DNA methylation profiles at birth and child ADHD symptoms. *J. Psychiatr. Res.* 49, 51–59 (2014).

53 Moser D, Ekawardhani S, Kumsta R *et al.* Functional analysis of a potassium-chloride co-transporter 3 (SLC12A6) promoter polymorphism leading to an additional DNA methylation site. *Neuropsychopharmacology* 34(2), 458–467 (2009).

54 Reimer J, Boniakowski E, Bachner C *et al.* When higher doses in opioid replacement treatment are still inadequate – association to multidimensional illness severity: a cohort study. *Subst. Abus. Treatment Prev. Pol.* 9, 13 (2014).

55 Lechner M, Fenton T, West J *et al.* Identification and functional validation of hpv-mediated hypermethylation in head and neck squamous cell carcinoma. *Genome Med.* 5(2), 15 (2013).

56 Reinius LE, Acevedo N, Joerink M *et al.* Differential DNA methylation in purified human blood cells: Implications for cell lineage and studies on disease susceptibility. *PLoS ONE* 7(7), e41361 (2012).

57 Davies MN, Volta M, Pidsley R *et al.* Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. *Genome Biol.* 13(6), R43 (2012).

58 Dobrinas M, Crettol S, Oneda B *et al.* Contribution of CYP2B6 alleles in explaining extreme (S)-methadone plasma levels: a CYP2B6 gene resequencing study. *Pharmacogenet. Genomics* 23(2), 84–93 (2013).

59 Oneda B, Crettol S, Jaquenoud Sirot E, Bochud M, Ansermot N, Eap CB. The P450 oxidoreductase genotype is associated with CYP3A activity *in vivo* as measured by the midazolam phenotyping test. *Pharmacogenet. Genomics* 19(11), 877–883 (2009).

60 Icick R, Peoc'h K, Ksouda K *et al.* OPRM1 polymorphism and lifetime suicide attempts among stabilized, methadone-maintained outpatients. *Psychiatry Res.* 218(1–2), 259–260 (2014).

61 Hajj A, Ksouda K, Peoc'h K *et al.* KCNH2 polymorphism and methadone dosage interact to enhance QT duration. *Drug Alcohol Depend.* 141, 34–38 (2014).

62 Icick R, Peoc'h K, Karsinti E *et al.* A cannabinoid receptor 1 polymorphism is protective against major depressive disorder in methadone-maintained outpatients. *Am. J. Addict.* 24(7), 613–620 (2015).

63 Sakae DY, Marti F, Lecca S *et al.* The absence of VGLUT3 predisposes to cocaine abuse by increasing dopamine and glutamate signaling in the nucleus accumbens. *Mol. Psychiatry* 20(11), 1448–1459 (2015).

64 Eap CB, Crettol S, Rougier JS *et al.* Stereoselective block of herg channel by (S)-methadone and QT interval prolongation in CYP2B6 slow metabolizers. *Clin. Pharmacol. Ther.* 81(5), 719–728 (2007).

65 Uehlinger C, Crettol S, Chassot P *et al.* Increased (R)-methadone plasma concentrations by quetiapine in cytochrome P450s and ABCB1 genotyped patients. *J. Clin. Psychopharmacol.* 27(3), 273–278 (2007).