

Differential DNA methylation in experienced meditators after an intensive day of mindfulness-based practice: Implications for immune-related pathways

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ABSTRACT

The human methylome is dynamically influenced by psychological stress. However, its responsiveness to stress management remains underexplored. Meditation practice has been shown to significantly reduce stress level, among other beneficial neurophysiological outcomes. Here, we evaluated the impact of a day of intensive meditation practice ($t_2 - t_1 = 8$ h) on the methylome of peripheral blood mononuclear cells in experienced meditators ($n = 17$). In parallel, we assessed the influence of a day of leisure activities in the same environment on the methylome of matched control subjects with no meditation experience ($n = 17$). DNA methylation profiles were analyzed using the Illumina 450 K beadchip array. We fitted for each methylation site a linear model for multi-level experiments which adjusts the variation between t_1 and t_2 for baseline differences. No significant baseline differences in methylation profiles was detected between groups. In the meditation group, we identified 61 differentially methylated sites (DMS) after the intervention. These DMS were enriched in genes mostly associated with immune cell metabolism and ageing and in binding sites for several transcription factors involved in immune response and inflammation, among other functions. In the control group, no significant change in methylation level was observed after the day of leisure activities. These results suggest that a short meditation intervention in trained subjects may rapidly influence the epigenome at sites of potential relevance for immune function and provide a better understanding of the dynamics of the human methylome over short time windows.

1. Introduction

There is growing evidence that the human epigenome is influenced by psychological stress. In particular, stressful life events have been shown to lead to long-lasting methylation at the glucocorticoid receptor gene and other methylation marks across the genome (Burns et al., 2018; McGowan and Roth, 2015). Such epigenetic effects may mediate the embodiment of stressful life events and contribute to their physiological and behavioral outcomes, such as the persistent cognitive

alterations, activation of the HPA axis and increased risk for psychopathology and chronic diseases (Brown et al., 2019; McEwen, 2017).

Fewer studies have investigated the extent to which stress management shapes the human epigenome, and may counterbalance these deleterious stress-induced epigenetic effects. Meditation, a family of practices based on attentional and emotional regulation (Lutz et al., 2008), has been shown to significantly reduce stress (Pascoe et al., 2017), among other beneficial outcomes at the emotional and cognitive levels (Barnhofer, 2019; Dahl et al., 2015; Klimecki et al., 2019; Luders

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and Kurth, 2019), as well as physiological and cellular levels (Black and Slavich, 2016; Buric, 2017; Conklin et al., 2019; Kaliman, 2019). In particular, the modulation of inflammatory pathways is an increasingly reported outcome of meditation-based interventions (MBIs). In the last 10 years, several studies have explored the impact of MBIs (i.e. mindfulness, meditation, yoga, Tai Chi, Qigong, relaxation response, and breath regulation) on gene expression in clinical and non-clinical populations. A recent review (Buric, 2017) highlighted the consistent downregulation of proinflammatory genes and pathways across these studies, in particular those dependent on the transcription factor *NF- κ B*, a key mediator of inflammation which has been shown to be upregulated by psychosocial stress (Bierhaus et al., 2003; Bekhbat et al., 2017).

Despite the growing understanding of the neurophysiological bases of meditation, few studies have investigated whether MBIs shape the epigenome. In this context, cross-sectional studies have shown that blood cells from long term meditators exhibit a trajectory of epigenetic ageing different from age-matched meditation-naïve controls, with a slowdown of the epigenetic clock as the number of years of practice increases (Chaix et al., 2017), and epigenetic changes in pathways related to common diseases and inflammatory signaling (García-Campayo et al., 2018). In addition, a longitudinal study in meditation trained subjects showed that a day of intensive mindfulness meditation induced a decrease in the expression level of histone deacetylase genes, as well as significant changes in histone acetylation levels, when compared to an active control group of meditation-naïve subjects engaged in leisure activities in the same environment (Kaliman et al., 2014). Consistent with the emerging anti-inflammatory role of HDAC inhibitors (HDACi) (Gatla et al., 2019), the decrease in HDAC gene expression in the meditation group was concomitant with a significant downregulation of proinflammatory genes *Receptor Interacting serine/threonine Kinase 2* (*RIPK2*) and *Cyclooxygenase-2* (*COX2*), which are regulated by HDACi in diverse cell systems (Tong et al., 2004; Roger et al., 2019).

Because histone deacetylases and histone modifications play a key role in epigenetic regulation, these findings raise the possibility that the meditative practice may rapidly modulate the human epigenome and influence clinically relevant signaling pathways. To test this hypothesis, we performed the first methylome-wide longitudinal study in peripheral blood mononuclear cells (PBMCs) from the same group of meditators and controls analyzed in our previous study (Kaliman et al., 2014), using high-throughput DNA methylation data.

2. Results

We obtained paired PBMCs samples from 18 meditators and 20 controls, before (t_1) and after (t_2) 8 h of a mindfulness meditation session for the meditators, and 8 h of leisure activities in the same environment for the meditation-naïve controls. DNA methylation profiles at 485,512 sites across the genome were analyzed for all samples using the Illumina 450 K beadchip array. After quality filtering, we retained a dataset comprising 414,717 methylation sites for 17 meditators and 17 controls individuals at t_1 and t_2 . The normalization procedure to remove technical noise and to maximize signal detection is described in detail in [Supplementary note 1](#). To correct methylation values for heterogeneity in blood cell types proportions, cell counts were estimated from methylation levels for each sample using a validated regression calibration algorithm (Accomando et al., 2014; Jaffe and Irizarry, 2014). No significant difference in blood cell types proportions was observed between t_1 and t_2 , nor between controls and meditators ([Supplementary Fig. 1](#), t. test p-values greater than 0.05 for both tests). Cell types proportions were taken into account in all subsequent analyses, along with gender and age.

2.1. Identification and localization of DMS

We fitted for each site a linear model for multi-level experiments

which models, at the same time, the variation in methylation profiles between t_1 and t_2 and the variation between meditators and controls. Notably, it adjusts the variation between t_1 and t_2 for baseline differences. No significant basal differences in methylation profiles was observed between meditators and controls after multiple testing correction (ie FDR adjusted P-value below 0.05). In the meditation group, we detected 2813 sites exhibiting differential methylation after the intervention with a FDR adjusted P-value below 0.05. After applying a more stringent criteria for differential methylation detection (a FDR adjusted P-value below 0.05 and a difference in methylation level of at least 3%), 61 sites were found to be differentially methylated after the meditation intervention ([Fig. 1](#)). In the control group, only one methylation site exhibited differential methylation after the intervention with a FDR adjusted P-value below 0.05 ([Supplementary Fig. 2](#)), however the difference in methylation level was below 3%. The top 5000 CpGs in each group with the highest signal of differential methylation after the intervention, ranked according to their FDR adjusted p-value, are listed in [Supplementary Tables 1 and 2](#).

Out of the 61 meditation-sensitive DMS, 57 showed an increased methylation level after the meditation intervention. The 61 meditation-sensitive DMS were distributed across all chromosomes, with the exception of chromosomes 9, 15, 18, 20 and 21 ([Fig. 2A](#)). Fifty-three of the 61 meditation-sensitive DMS were located in known genes (in total 55 genes, as two DMS localized in two separate genes). [Supplementary Table 3](#) summarizes the reported function in Uniprot database of each of these 55 genes. We observed that meditation-sensitive DMS were significantly more often found in proximal promoters and first exons of genes (p-value less than 0.05) than under the null hypothesis of random genomic localization ([Fig. 2B](#) and [Supplementary Table 4](#)). Two-thirds of gene promoters exhibit CpG islands – a cluster of high density CpG known to play a key role in the regulation of genic expression. Consequently, we investigated whether the meditation-sensitive DMS were preferentially located in CpG islands or in shores (from 0 to 2 kb from islands, also known to exhibit tissue and condition specific methylation patterns), shelves (from 2 to 4 kb from islands) or open seas (more than 4 kb from islands). We observed that meditation-sensitive DMS were significantly more often found in CpG Islands (p-value $< 10^{-4}$), and less often found in open seas (p-value $< 10^{-4}$) than under the null hypothesis of random genomic localization ([Fig. 2B](#) and [Supplementary Table 4](#)).

2.2. Functional analysis of meditation-sensitive DMS

We tested whether specific gene ontology categories or KEGG pathways were significantly enriched in meditation-sensitive DMS. Four KEGG pathways exhibited a significant enrichment in meditation-sensitive DMS after multiple testing correction ([Table 1](#)): two of these categories were related to fatty acid metabolism, comprising genes *ACADM*, *CPT1A* and *HSD17B4*. Other categories significantly enriched were related to RNA transport (with genes *SAP18*, *EIF1B*, *NCBP2*), and to the Fanconi anemia pathway (with genes *APITD1* and *ERCC1*), which is involved in the preservation of the genome stability. The top 5 GO biological processes, were linked to betaine metabolism, fatty acid metabolism and immunity, although none of them showed significant enrichment in meditation-sensitive DMS after multiple testing correction ([Supplementary Table 5](#)).

To investigate further the biological functions of genes associated to meditation-sensitive DMS, we performed a gene-gene interacting network using the STRING database, which integrates known and predicted protein–protein interactions, including physical (direct) and functional (indirect) associations ([Supplementary Fig. 3](#)). This analysis supported results from the KEGG pathways analysis. In particular, *ACADM*, *CPT1A* and *HSD17B4* emerged as a functional cluster involved in the beta-oxidation pathway within fatty acid metabolism. The largest cluster comprised *APITD1*, *ERCC1*, *PHF21A*, *SAP18*, *HNRNP1*, *NCBP2*, *EIF4E3* and *GPR27* genes which are involved in DNA repair (*APITD1*

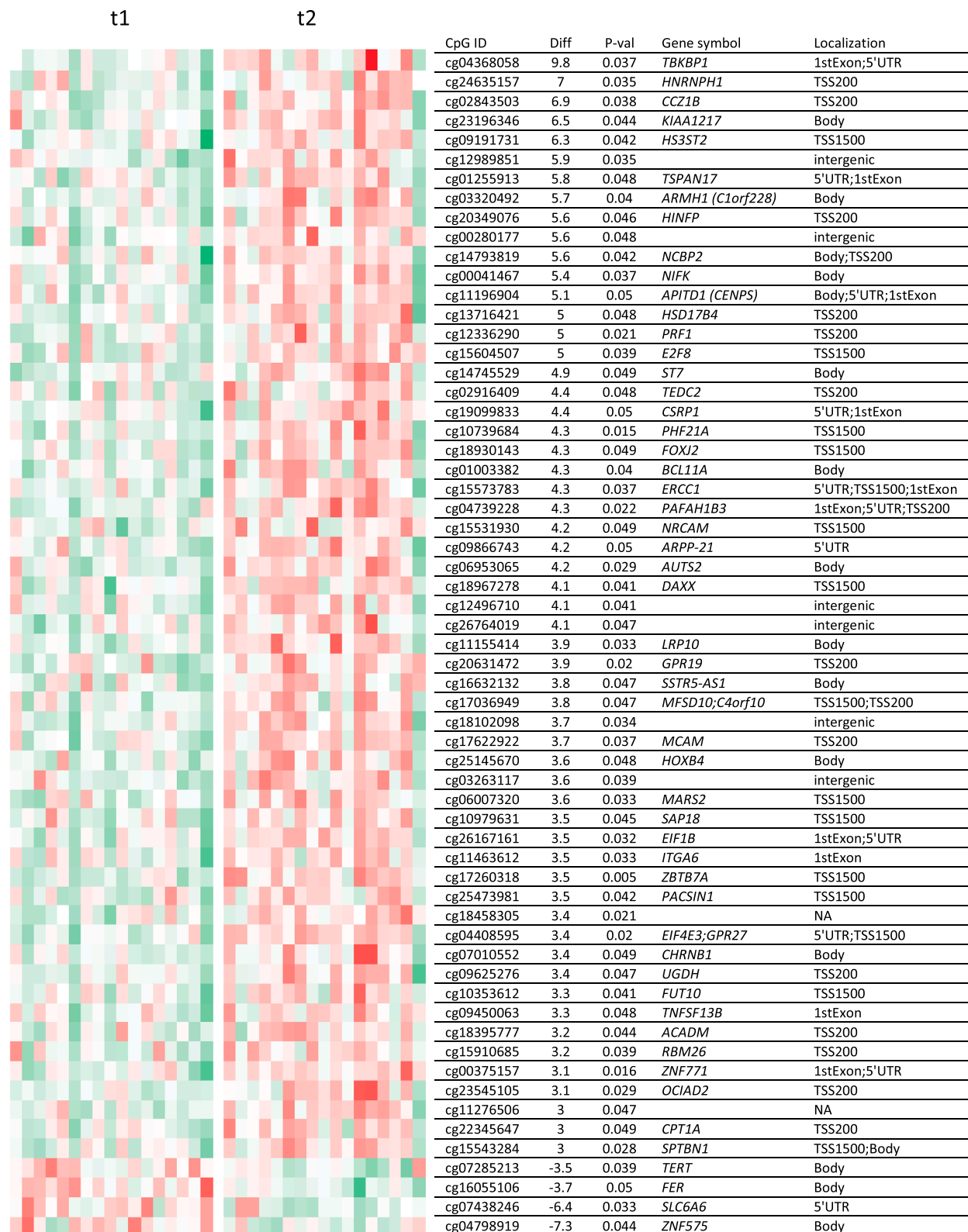


Fig. 1. Heatmap of meditation-sensitive differentially methylated sites (DMS). 61 sites showed methylation levels either significantly increased or decreased (after multiple testing correction) by at least 3% in the meditators group after the meditation intervention (t_2) in comparison to before the intervention (t_1). Each column is one individual (in t_1 on the left and in t_2 on the right), each line is one meditation-sensitive DMS. Sites with the largest increase in methylation are presented at the top (e.g. the gene *TBKBP1* exhibited the largest increase). The color indicates the relative methylation level, from low (green) to high (red). CpG ID: Illumina CpG ID, Diff: difference in methylation level between t_2 and t_1 , P-val: FDR adjusted P-value, TSS200: proximal promoter (less than 200 bp upstream from the transcription start site), TSS1500: distal promoter (from 1,500 to 200 bp upstream from the transcription start site). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

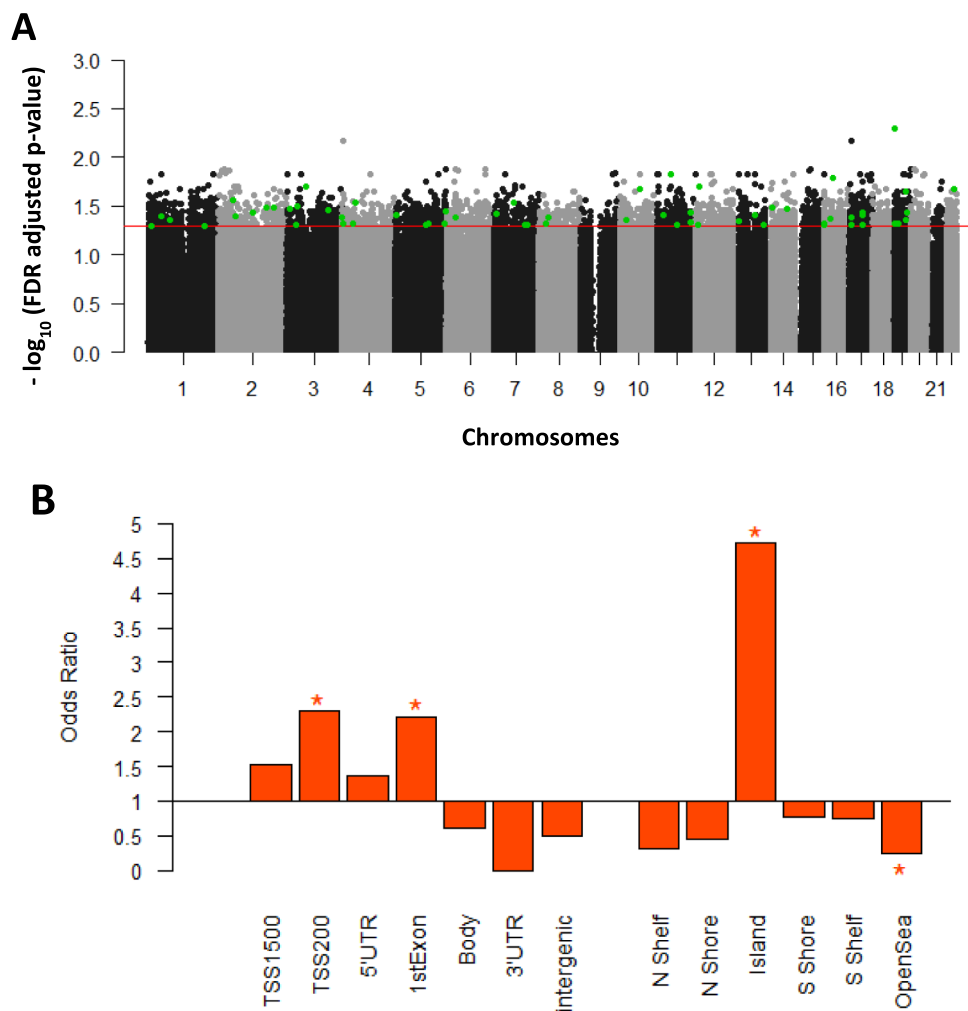


Fig. 2. Distribution of the 61 meditation-sensitive DMS across the genome. A) In this Manhattan plot, the black and grey points indicate the FDR adjusted p-value for each of the 414,717 tested CpG and the red line marks the 5% threshold for this FDR adjusted p-value (2813 sites exhibit a FDR adjusted p-value below 0.05). The green points indicate the 61 meditation-sensitive DMS (sites that passed this 5% FDR adjusted p-value threshold and exhibit a minimum 3% difference in methylation change during the meditation intervention) B) This figure shows the enrichment of different genomic regions in meditation-sensitive DMS: distal promoters (TSS1500), proximal promoters (TSS200), 5'UTRs, first exons, gene bodies, 3'UTRs, intergenic regions, CpG islands, shores, shelves and open seas (*chi-square test p-value < 0.05, see supplementary Table 4 for all p-values). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and *ERCC1* were associated to the Fanconi anemia pathway in the KEGG analysis), in chromatin remodeling and in RNA metabolism and protein translation (Costello et al., 2017; Volpon et al., 2013). STRING analysis also revealed 3 additional gene-gene interactions: *NRCAM* and *SPTBN1*, involved in axonal growth and neurotransmission (Amor et al., 2017); *DAXX* and *TERT*, involved in telomerase regulation and telomeres maintenance (Tang et al., 2015); *ITGA6* and *MCAM*, involved in cell adhesion (Shirali et al., 2018).

2.3. Motif enrichment analysis

We performed a motif enrichment analysis (McLeay and Bailey, 2010), considering a flanking sequence of 150 bp (± 75 bp) around each meditation-sensitive DMS. Five motifs were found significantly enriched, corresponding to binding sites for *KLF15*, *EGR1*, *EGR2*, *SP3* and *SP4* transcription factors which are involved in immune response and inflammation as well as other biological processes. Table 2 presents the enriched motifs and corresponding transcription factors as well as references relevant in the context of immunity and inflammation. Notably, *KLF15* (Krüppel-like factor 15) regulates vascular inflammation through the interaction with *NF- κ B*, and its expression is directly induced by glucocorticoids (McConnell and Yang, 2010; Lu et al., 2013). *SP3* (Specificity protein 3) controls the expression of inflammation-related molecules such as the anti-inflammatory cytokine *IL-10* and the pro-inflammatory molecule *COX-2* (Lee et al., 2006; Tone et al., 2019). When considering in this analysis a longer flanking sequence around each DMS (500 bp), the same 5 motifs were significantly enriched,

along with 29 other motifs (the top 10 enriched motifs and their biological activity are summarized in Supplementary Table 6).

3. Discussion

While it has been reported by many studies that the human methylome is dynamically influenced by psychological stress, its responsiveness to stress reduction remains poorly explored. Here, we performed a methylome-wide scan to detect differential methylation in response to an intense 8 h meditation practice in subjects with long-term meditation training. We found that the day long intensive practice of mindfulness meditation impacted the methylation profile of the participants at 61 CpG sites (over 3% change in methylation level and p-value below 0.05 after multiple testing correction). No significant change in methylation profiles was seen in the control group in response to the leisure day. In addition, there was no significant basal difference between the meditators and the controls in terms of methylation profiles. This corroborates the previously reported absence of basal difference between the two groups in terms of transcriptomic profiles (Kaliman et al., 2014).

We analyzed the biological pathways associated with the 61 meditation-sensitive DMS through KEGG pathway enrichment analysis (Table 1), STRING analysis (Supplementary Fig. 3) and Motif enrichment analysis (Table 2). A previous transcriptome and protein level analysis performed on the same samples highlighted that this intervention led to a decrease in HDAC gene expression and to a significant downregulation of proinflammatory genes (Kaliman et al., 2014).

Table 1
KEGG pathways significantly enriched in meditation-sensitive DMS. This table shows the KEGG pathways that are significantly enriched (after FDR multiple testing correction) in meditation-sensitive DMS (considering the 61 DMS with at least 3% of change in methylation level after the meditation intervention). N: number of genes in the pathway, DM genes: list of genes in the pathway containing meditation-sensitive DMS.

KEGG path:	Pathway	N	FDR adjusted P-value	DM genes	Full name; function (Uniprot database functional annotation)
hsa01212	Fatty acid metabolism	52	0.003	ACADM CPT1A HSD17B4	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial/Involved in the electron transfer to the main mitochondrial respiratory chain Carnitine O-palmitoyltransferase 1, liver isoform/Catalyzes the transfer of the acyl group of long-chain fatty acid-CoA conjugates onto carnitine Peroxisomal multifunctional enzyme type 2/Bifunctional enzyme acting on the peroxisomal beta-oxidation pathway for fatty acids
hsa03013	RNA transport	149	0.016	SAP18 EIF1B NCBP2	Histone deacetylase complex subunit SAP18/Enhances the ability of SIN3-HDAC1-mediated transcriptional repression Eukaryotic translation initiation factor 1b/Translation machinery Nuclear cap-binding protein subunit 2/Component of the cap-binding complex, involved in pre-mRNA splicing and translation regulation
hsa00071	Fatty acid degradation	42	0.033	ACADM CPT1A	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial/Involved in the electron transfer to the main mitochondrial respiratory chain Carnitine O-palmitoyltransferase 1, liver isoform/Catalyzes the transfer of the acyl group of long-chain fatty acid-CoA conjugates onto carnitine
hsa03460	Fanconi anemia pathway (DNA repair)	49	0.048	APTD1 ERCC1	Centromere protein 5/Involved in the response to DNA damage, cellular resistance to DNA cross-linking drugs, and prevention of chromosomal breakage DNA excision repair protein ERCC-1/Non-catalytic component of a structure-specific DNA repair endonuclease

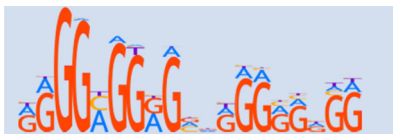




Consistent with these previous findings, here we detected differential methylation in biological pathways of relevance for the inflammatory and immune systems after the meditation intervention. In particular, the 61 meditation-sensitive DMS were significantly enriched in binding sites for five transcription factors (*KLF15*, *EGR1*, *EGR2*, *SP3*, *SP4*) involved in immunity and inflammation among other biological processes (Mcconnell and Yang, 2010; Lu et al., 2013; Lee et al., 2006; Tone et al., 2019; Sheehan et al., 2019; Li et al., 2012; Miao et al., 2017). The enrichment in the binding site *SP3* (Specificity protein 3) is particularly interesting for our study as this transcription factor controls the expression of the pro-inflammatory molecule cyclooxygenase-2 *COX2* (Lee et al., 2006; Tone et al., 2019). Indeed, a previous report on the same samples analyzed here has shown that the daylong meditation intervention significantly decreased the gene and the protein expression of *COX2* (Kaliman et al., 2014). As we did not detect significant changes in the methylation level of *COX2* gene, these data suggest that the meditation practice may modulate the expression of this gene through changes in the activity of specific regulatory transcription factors, rather than through methylation changes. This corroborates a recent study suggesting that changes in transcription levels may precede changes in methylation level (Pacis et al., 2019). In addition, the greatest methylation change (in terms of magnitude) in response to the intervention localized in the *TBKBP1* gene which is involved in the *TNF-α/NF-κB* pathway (Fig. 1). This pathway is activated in blood cells under conditions of acute and chronic psychological stress (Pace et al., 2006; Miller et al., 2008; Miller et al., 2009). Notably, a decrease in *NF-κB* activity seems to be a consistent genomic fingerprint of mind-body therapies, that may underlie the potential anti-inflammatory effects of these practices (Black and Slavich, 2016; Buric, 2017). At least two other methylation sensitive DMS are located in genes previously associated with immune and inflammatory pathways: *TNFSF13B* that codes for a cytokine belonging to the *TNF* family (Schneider et al., 1999); and *PRF1*, whose expression and methylation level in blood cells was previously shown to respond to psychological stress exposure, and in particular to the Trier Social Stress Test (TSST) (Falkenberg et al., 2013). In addition, two intergenic meditation-sensitive DMS, cg12989851 and cg12496710, were previously shown to have their methylation level in blood associated with inflammatory disorders (Imgenberg-Kreuz et al., 2016; Liu et al., 2013).

Consistent with the role of the ANS and the HPA axis in the regulation of energy balance (Peckett et al., 2011), lipid metabolism was the pathway most significantly enriched according to the KEGG analysis, with genes *ACADM*, *CPT1A* and *HSD17B4*. These three genes also emerged as a cluster in the STRING analysis. Our findings suggest that this metabolic pathway might have been modulated by the stress reducing effects associated with meditation (Pascoe et al., 2017). Immune cells use lipids as a source of energy, as any other cell, by degrading fatty acids in a process termed beta-oxidation. However, lipid metabolism is also currently viewed as a central switch regulating T cell fate decisions. Fatty acid oxidation seems to guide specific T cell fates and functions including the induction of CD4 + regulatory T and CD8 + memory T cells (Lochner et al., 2015). Here we analyzed PBMCs, which are composed of lymphocytes (T cells, B cells, and NK cells) in the range of 70–90 % (K.C.R., 2015). Previous studies have reported significant increases in antibody titers to influenza vaccine (Davidson et al., 2003) and effects on CD4 + T lymphocytes in HIV-1 infected adults (Creswell et al., 2009), in response to MBIs. Based on the data presented here, we hypothesize that immune function improvement by stress reduction strategies could be mediated at least in part by methylation changes in specific fatty acid metabolism genes. This hypothesis warrants further investigation.

The pathway most consistently differentially methylated (according to the KEGG, STRING and motif enrichment analyses) after the meditation intervention involves DNA repair and response to DNA damage. More precisely, the Fanconi anemia pathway, that preserves genome stability (Moldovan and D’Andrea, 2009), was significantly enriched in

Table 2

Transcription factor motif enrichment in meditation-sensitive DMS. DNA sequences flanking the 61 meditation-sensitive DMS (± 75 bp) were used to identify enriched motifs using the AME suite package (McLeay and Bailey, 2010), considering an E-value cut-off of 0.05. Logo plots of the binding site motifs, transcription factors predicted to bind to each motif, Bonferroni adjusted p-value, E-value, the number of DMS where the motif is present, and reported functions based on Uniprot database and related bibliography are shown.

Logo	Rank	Symbol	adj. P-value	E-value	N° of DMS	Full name; function
	1	KLF15	4.42e-6	1.78e-3	16	Krüppel-like factor 15 ; involved in cellular glucose homeostasis, stress response and inflammation. <i>KLF15</i> regulates vascular inflammation through the interaction with <i>NF-κB</i> , and its expression is directly induced by glucocorticoids (McConnell and Yang, 2010; Lu et al., 2013).
	2	EGR1	7.82 e-6	3.14 e-3	23	Early Growth Response gene 1 ; involved in many biological processes, such as response to DNA damage, immunity, and inflammatory responses
	3	SP3	8.91 e-6	3.58 e-3	17	Specificity protein 3 ; involved in response to DNA damage, immunity, hematopoiesis, and controls the expression of inflammation-related molecules such as the anti-inflammatory cytokine <i>IL-10</i> and the pro-inflammatory molecule <i>COX-2</i> (Lee et al., 2006; Tone et al., 2019)
	4	SP4	1.59e-5	6.39 e-3	16	Specificity protein 4 ; involved in inflammatory and neuropathic persistent pain states (Sheehan et al., 2019) and in dendrite patterning and neurotransmission (Li et al., 2012)
	5	EGR2	4.32 e-5	1.74e-2	22	Early Growth Response gene 2 ; involved in immunity and inflammatory processes (Miao et al., 2017; Pacis et al., 2019), as well as various neuropathies.

the KEGG analysis, with genes *ERCC1* and *APTD1*. This pathway was also highlighted in the largest cluster that emerged in STRING analysis. Moreover, about a third of the 61 meditation-associated DMS are located in and nearby binding sites for transcription factors *EGR1* and *SP3* that play a role not only in immune function but also in the response to DNA damage. This observation of differential methylation in the DNA damage response pathway may be related to the stress reducing effects of the meditation practice, as previous reports suggest an association between exposure to psychological stress and DNA damage (Flint et al., 2007; Gidron et al., 2006). Moreover, our study identified meditation-induced DMS associated with cell aging. Strikingly, one of them localized in the gene body of *TERT* (telomerase reverse transcriptase), that codes for a subunit of the telomerase enzyme. This enzyme elongates telomeres, which are nucleoproteins complexes protecting the ends of chromosomes from instability and degradation. A cluster linking *TERT* to *DAXX* gene, shown to be involved in telomere maintenance, also emerged in the STRING analysis. Greater overall telomere attrition predicts mortality and aging-related diseases and both telomeres length and telomerase activity are sensitive to psychological stress (Blackburn et al., 2015). On the other hand, there is emerging evidence that MBIs may modulate telomerase activity, and possibly, increase telomere length (Conklin et al., 2019). These results suggest that meditation practice may elicit epigenetic changes that are associated with telomere biology, and more generally biological aging. Our findings showing enrichment in DMS associated with DNA damage and cell aging are particularly interesting in the context of PBMCs. Indeed, increased DNA damage and telomere attrition in the immune system lead to the accumulation of genomically damaged and senescent cells (Keenan and Allan, 2019) and cellular senescence seems to be associated with low grade chronic inflammation through the immune cell acquisition of the senescence-associated secretory phenotype (López-Otín et al., 2013; Sikora et al., 2011). Taken together, these data suggest that MBIs may

help prevent or delay immune system aging at least in part through epigenetic regulation of DNA repair mechanisms and telomere biology. These findings corroborate our recent observation of different epigenetic aging trajectories in PBMCs between long term meditators and controls, as well as a slowdown of their epigenetic clock with the number of years of meditation practice (Chaix et al., 2017).

Another KEGG pathway significantly enriched among the 61 meditation-sensitive DMS was related to RNA transport, and includes genes *SAP18*, *EIF1B*, *NCBP2*. We also observed that the largest cluster that emerged in the STRING analysis comprises *NCBP2*, *EIF4E3* and *GPR27* genes that are related to RNA metabolism and protein translation. In addition, this largest cluster in the STRING analysis is also enriched in genes involved in chromatin remodeling and in epigenetic regulation, in particular *PHF21A* (Garay et al., 2016) and *SAP18* (Zhang et al., 1997) that recruit histone deacetylase (HDAC)-containing complexes. This suggests that chromatin recruitment and the activity of histone deacetylases might be sensitive to meditation practice. Taking into account the role of histone acetylation in inflammation (Gatla et al., 2019), our data suggest an epigenetic basis for the anti-inflammatory effects of MBIs and are consistent with our previous data showing a concomitant regulation of histone acetylation and inflammatory pathways by meditation practice (Kaliman et al., 2014).

STRING analysis revealed a cluster of two genes associated with cell adhesion (*MCAM* and *ITGA6*) which could be related to vascular regeneration, as *MCAM* is more specifically involved in angiogenesis. Supporting these findings, the expression levels of *ITGA6* and *MCAM* have been shown to be modified during wound repair and more specifically vascular regeneration (Shirali et al., 2018) and a recent study suggested that mindfulness might have beneficial effects on early stages of wound healing (Meesters et al., 2018).

Finally, several meditation-sensitive DMS relate to neurological processes. In particular, a gene cluster involved in axonal growth and

neurotransmission was highlighted in the STRING analysis. More generally, a number of genes that contain meditation-sensitive DMS have been previously reported as potential blood biomarkers of mental health: this is the case for *PACSN1* (Cecil et al., 2016) and *SPTBN1* (Liu et al., 2018) associated with addiction related behavior; *GPR19* associated with depression (Van Assche et al., 2017); *AUTS2* associated with autism (Wong et al., 2014). Moreover, about 25% of the meditation-sensitive DMS correspond to binding sites for transcription factor *SP4*, involved in dendrite patterning and neurotransmission (Sun et al., 2015). Because of the tissue-specific nature of epigenetic processes, these findings need to be interpreted with caution.

Most of the 61 meditation-sensitive sites exhibited an increase in methylation level and were located upstream of genes (promoters and first exons) and in CpG islands. DNA methylation generally plays a repressing role on gene expression, especially when located at CpGs upstream of genes. Consequently, some of the methylation changes we report here may be associated with a decreased gene expression. We interpret this skewed landscape of increased methylation at sites located upstream of genes as a methodological lack of sensitivity to detect decreases in methylation at CpG islands which are known to depict low basal methylation levels (Hernando-Herraez et al., 2015; Jones and Takai, 2001). Similarly, our study did not detect significant changes in methylation profiles in the control group after the leisure day. The lack of participant randomization due to the fact that we compared meditation experts with unexperienced subjects, may, at least in part, explain this result. Moreover, while the meditation group was instructed to follow the same mental-training practices throughout the intervention, participants in the control group could voluntarily choose between a variety of activities (i.e. resting, reading, watching documentaries or playing computer games). Such heterogeneity of leisure activities may have introduced a variability decreasing the statistical power to detect specific changes on DNA methylation in the control group. These observations further highlight the need to design future studies with active control interventions that are structurally comparable to the meditation interventions (Davidson and Kaszniak, 2015). In addition, in the future, it will be important to determine if a one-day intervention in otherwise naïve participants would produce any of the effects we observed in the current study performed in long term meditators.

To conclude, using stringent criteria for DMS identification, our study shows that a short meditation intervention in trained subjects rapidly influenced the methylome at sites of potential clinical relevance, related to the transcriptional regulation of the inflammation response, immune cell metabolism, DNA repair, cell aging, RNA metabolism, protein translation, cell adhesion and neurotransmission. Whereas methylation marks are usually considered to be relatively stable, our observations strengthen a growing body of research in humans and murine models reporting fast methylation changes, in particular in response to acute stress (Falkenberg et al., 2013; Mifsud et al., 2017; Saunderson et al., 2016; Rodrigues et al., 2015; Unternaehrer et al., 2012). Altogether, these studies depict the complexity of methylome dynamics, combining both stable and environmentally labile marks. The relationship between the fast epigenetic changes elicited by this daylong meditative practice in experienced practitioners and the previously reported long lasting effects of this practice remains to be investigated. Future randomized controlled studies with larger sample sizes, active control groups and long-term follow-ups are required to validate the findings of this initial study and to explore their health-related potential.

4. Material and methods

4.1. Participants and interventions.

Long-term meditators ($n = 19$) and meditation-naïve controls ($n = 21$) were studied. The demographic characteristics of the groups and interventions are described more extensively in (Kaliman et al.,

2014). Both groups showed similar distributions of age (controls: 50.38 ± 8.96 ; meditators: 49.89 ± 11.18), gender (controls: 12F and 9 M; meditators: 11 F. 8 M), race (controls: 95.23% Caucasian; meditators: 84.21% Caucasian) and body-mass index (controls: 23.55 ± 3.76 ; meditators: 24.16 ± 3.51). Their peripheral blood mononuclear cells (PBMC) were obtained at 8 am (Time 1) and at 4 pm (Time 2), respectively before and after an intensive day of mindfulness meditation for the meditators, and a day of leisure activities in the same environment for the controls. The UW-Madison Health Sciences Internal Review Board approved this study and all participants provided written informed consent. The criteria for inclusion in the meditators group were i) a daily meditation practice spanning a minimum of 3 years, ii) at least 30 min of daily sitting meditation and iii) a minimum of 3 intensive retreats lasting 5 or more days. All experienced meditators practiced both standard mindfulness-related meditations (e.g. Vipassana and concentration meditations) and compassion-related meditations. The controls had no prior meditation experience. The interventions for the meditators and the control group were matched in terms of physical activity. The meditation intervention largely overlapped in terms of contents with the day-long session of the Mindfulness-Based Stress Reduction program (MBSR), that is routinely used in North-America hospitals (Kabat-Zinn, 1982). The controls were engaged in intentional activities, such as reading, watching documentaries or playing computer games, and walking.

4.2. Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were obtained from each participant and PBMCs were isolated, as described in Kaliman et al. (Kaliman et al., 2014). DNA was isolated using QIAamp DNA Blood Mini Kit, and stored at -80°C until processing. Samples from two participants from the original study could not be analyzed due to insufficient DNA quality.

4.3. Genome-wide DNA methylation profiling

We used the Infinium HumanMethylation450 beadchip array (Bibikova et al., 2011) to examine methylation levels at 485,512 sites for 79 samples, including 20 pre-intervention (t_1) controls, 20 post-intervention (t_2) controls, 18 pre-intervention (t_1) meditators, 18 post-intervention (t_2) meditators, as well as 3 technical replicates. DNA methylation data from all participants were generated at the same time, in a single batch. DNA samples from both groups were randomized across the arrays.

4.4. Sample filtering, probe filtering and data normalization procedure

Sample filtering, probe filtering and data normalization procedure are presented in Supplementary note 1.

4.5. Accounting for heterogeneity in cell subtypes

The DNA samples have been extracted from a mixture of cell types (PBMC). Consequently, it is crucial to take into account the heterogeneity in cell types proportions across samples in our analyses, since this heterogeneity may introduce a variation in the data (Houseman et al., 2012). To estimate the relative proportions of CD8 + T cells, CD4 + T cells, NK cells, B cells, and monocytes for each participant, we used the estimate Cell Counts function from the R Bioconductor package Minfi (Aryee et al., 2014), a regression calibration algorithm for deconvoluting heterogeneous tissues (Jaffe and Irizarry, 2014; Ritchie et al., 2015). The estimated cell counts were rescaled to 1 and were taken into account as covariates in all subsequent analyses. The probes that were used to predict cell counts proportions were removed from the data set for subsequent analyses, yielding a final set of 414,717 probes.

4.6. Determination of differentially methylated sites (DMS) after a day of intensive meditative practice

Sites differentially methylated between meditators and controls and between t_1 and t_2 were identified statistically by fitting at each site a linear mixed model (M -values $\sim Mt_1 + Mt_2 + Ct_1 + Ct_2 + \text{sex} + \text{age} + \text{cell type proportions} + \text{error}$), with the R bioconductor limma package (Benjamini and Hochberg, 1995). Mt_1 , Mt_2 , Ct_1 and Ct_2 are binary variables indicating whether data correspond to meditator (M), control (C), pre (t_1) or post (t_2) intervention. Individuals were treated as random effects. This model is specifically designed for multi-level experiments and allows comparing simultaneously two groups (here meditators and controls) and comparing paired samples (here two samples from the same individual, in t_1 and t_2). Crucially, it adjusts the detected variation between t_1 and t_2 for baseline differences in methylation profiles. Note that the paired test used to compare data within subjects is expected to be more powerful than the non-paired test used to compare data between subjects. We used a contrast matrix to identify sites with signals of differential methylation between meditators and controls and between t_1 and t_2 in meditators and in controls. Sites with a FDR adjusted P-value (M. Ashburner and Consortium, 2000) below 0.05 and a difference in methylation level between t_1 and t_2 of at least 3% were considered to be differentially methylated (DMS). This difference in methylation level was computed on β -values adjusted for age, gender and cell types proportions.

4.7. Genomic features of differentially methylated sites

To characterize the spatio-functional localization of DMS, we assessed whether particular genomic regions were enriched in meditation-sensitive DMS. For each site, we used the Illumina HumanMethy450 annotation table to identify DMS located within genic regions, as well as their precise genomic location using the UCSC_RefGene_Group column: into a distal promoter (TSS1500, from 1,500 to 200 bp upstream from the transcription start site), a proximal promoter (TSS200, less than 200 bp upstream from the transcription start site), a 5'UTR, a first exon, a gene body or a 3'UTR. Then, we used the Relation_to_Island column to identify the DMS located into CpG islands, shores (from 0 to 2 kb from islands), shelves (from 2 to 4 kb from islands) and open seas (more than 4 kb from islands). We assessed the enrichment of these regions among the identified DMS through the computation of an Odds-Ratio, defined as

$$OR = \frac{\left[\frac{P(R|DMS)}{P(\text{not } R|DMS)} \right]}{\left[\frac{P(R|\text{not } DMS)}{P(\text{not } R|\text{not } DMS)} \right]}$$

with R being 'in the region'. Significance of enrichment was tested for each region (over all meditators or all controls) using Chi-square test.

4.8. Gene ontology (GO) and KEGG analysis

We performed an analysis of the over-representation of gene ontology (GO) categories (Phipson et al., 2015) or KEGG pathways (Kyoto Encyclopedia of Genes and Genomes) among differentially methylated sites. We used the gometh function from the R bioconductor package missMethyl [81] which corrects for the differing number of probes per gene present on the array.

4.9. Protein-protein association representation by STRING database analysis

We constructed the protein-protein interaction network based on the list of DMS-associated genes found in this study using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) <https://string-db.org>.

4.10. Transcription factor motif discovery

The DNA sequences flanking the DMS found in the study were used to identify enriched motifs using the AME suite package (McLeay and Bailey, 2010), a part of the MEME Suite online platform. An E-value cut-off of 0.05 was considered to identify significantly enriched motifs. We ran two independent analyses, considering flanking sequences around each DMS of either 150 (± 75) or 500 (± 250) bp length.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2019.11.003>.

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