Mindfulness (2018) 9:1146–1159
https://doi.org/10.1007/s12671-017-0851-6

ORIGINAL PAPER

Epigenetic Response to Mindfulness in Peripheral Blood Leukocytes Involves Genes Linked to Common Human Diseases

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Published online: 1 December 2017

Abstract The benefits of mindfulness on human health are well-documented, but the mechanisms underlying these effects are not well-understood. Our aim was to identify molecular alterations related to mindfulness by profiling the epigenetic response in long-term meditators. We performed a genome-wide screen of DNA methylation by using the Illumina HumanMethylation450 platform in peripheral blood leukocytes from 17 long-term meditators and 17 matched controls. Top-ranked genes were validated by bisulfite cloning sequencing. Database for Annotation, Visualization, and Integrated Discovery and Ingenuity Pathways Analysis bioinformatic tools were used to characterize key processes underling the methylation changes. We found 64 differentially methylated regions (DMRs) in meditators compared to controls, corresponding to 43 genes. Most of the mindfulness-related DMRs (70.3%) were hypomethylated in meditators, and 23.4% of mindfulness-related DMRs clustered in telomeric chromosomal regions. Notably, almost half of the mindfulness-related DMRs (48.4%) involved genes linked to common human diseases, such as neurological and psychiatric disorders, cardiovascular diseases, and cancer. Lipid metabolism and atherosclerosis signaling pathway were significantly enriched in our set of DMRs. Functional in silico analysis also revealed tumor necrosis factor (TNF) and NF-κB signaling as crucial regulators of the mindfulness-related genes. Our study suggests that there is a consistent epigenetic response to long-term meditation practice in blood leukocytes with predominant loss of cytosine-phosphate-guanine methylation in distinct genomic regions, such as telomeres. Further research is warranted to confirm that the molecular response to mindfulness practice involves crucial transcriptional regulators related to a wide range of human common diseases, such as TNF and NF-κB.

Keywords Mindfulness · DNA methylation · Epigenetics · TNF · NF-κB

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12671-017-0851-6) contains supplementary material, which is available to authorized users.

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Mindfulness in the west has been used as a psychological therapy aimed at reducing stress by learning a structured, non-religious program that uses techniques based on meditation to develop a greater awareness of experience and thought processes. Mindfulness has been described as an awareness focused on the present moment, non-elaborative and non-judgmental, in which each thought, feeling, or sensation that arises in the attentional field is acknowledged and accepted as it is (Bishop et al. 2004; Segal et al. 2002; Shapiro and Schwartz 2000).

It is well-known that mindfulness improves mental and physical health, reduces negative affect, and enhances

adaptation to adverse or stressful situations (Grossman et al. 2004; Keng et al. 2011). An example of the effect of mindfulness on biological mechanisms and functions is the observation of immune changes in the blood and leukocytes after mindfulness mediation practicing, which has been systematically reviewed (Black and Slavich 2016). Among these changes, the 1970s Mindfulness-Based Stress Reduction (MBSR) program is related to a buffered CD4+ T lymphocyte decline (Creswell et al. 2009). In addition, some circulating proteins seem to be or show a trend to be decreased after a MBSR program, as is the case of C-protein reactive (CPR) (Creswell et al. 2012), or after mindful awareness practices (MAPs), such as interleukin 6 (IL-6) (Bower et al. 2015). However, these studies have been carried out after short-term mindfulness programs so long-term meditation effects on immunity need to be assessed.

Recently, it has been observed that practicing mindfulness and other meditation methods produces lasting morphological changes in the brain, such as an increase in region-specific cortical gray thickness, larger hippocampal dimensions, or white matter connectivity changes, among others (Fayed et al. 2013; Fox et al. 2014; Hernández et al. 2016; Kang et al. 2013; Luders et al. 2013; Tang et al. 2015;). In addition, meditation has been negatively associated with neurodegenerative conditions, such as Alzheimer's disease (Huang et al. 2016). Nevertheless, biological and molecular mechanisms underlying these effects remain largely unknown (Black and Slavich 2016).

Epigenetics may underlie some of these beneficial changes. Epigenetics is defined as the set of heritable biochemical modifications that regulate gene expression without changing the DNA sequence (Akbarian et al. 2013). Indeed, epigenetic modifications are the basis for gene-environment interactions, which in turn modulate the diversity of human phenotypes. Major epigenetic mechanisms of gene regulation include DNA methylation, histone post-translational modifications, and silencing action of non-coding RNAs. DNA methylation refers to the addition of a methyl group to the 5-carbon position of the cytosine-phosphate-guanine (CpG) sites, which are grouped in the genome forming the so-called CpG islands, mainly located at regulatory elements. Methylation status of CpG islands is associated with the transcriptional status of nearby or related genes (Suzuki and Bird 2008). DNA methylation patterns have an impact on gene expression in the human brain and blood (Horvath et al. 2012). On the other hand, aberrant DNA methylation patterns are involved in an increasing number of common diseases (Bergman and Cedar 2013; Heyn and Esteller 2012), including neurological and mental disorders. Genome-wide DNA screening is a helpful approach to identify DNA methylation alterations in the genome with no previous hypothesis. This approach may be achieved using different strategies and technologies (Bock 2012), the bisulfite microarrays, such as Infinium HumanMethylation450 BeadChip array from Illumina, being one of the most widely used technologies. This array explores more than 485,000 CpG sites in the genome, and once the data are processed and analyzed, it may reveal regulatory regions in the genome relevant to the issue or disease under study.

So far, little is known about epigenetic modifications induced by mindfulness practice. A recent study has shown that meditation decreases the expression of histone deacetylase genes, which are involved in chromatin remodeling and gene expression (Kaliman et al. 2014). Nevertheless, no studies have reported on how meditation influences DNA methylation patterns.

The aim of the present study was to profile genome-wide DNA methylation patterns in peripheral blood leukocytes (PBLs), isolated from long-term mindfulness practitioners compared to controls, in order to identify differentially methylated regions related to mindfulness practice and discover candidate molecular pathways underlying the biological effects of mindfulness. This kind of genome-wide approach is characteristically non-hypothesis-driven and allows the discovering of novel regions that do not need a previous knowledge to be tested.

Method

Participants

The study was designed as an observational, transversal case-control study. A collection of 17 long-term mindfulness meditators (MMs) was recruited from the Spanish Associations of Mindfulness and from the Master of Mindfulness of the University of Zaragoza. The comparison group, 17 control subjects, was recruited among healthy relatives and friends of the mindfulness meditators (MMs) who had a similar lifestyle. Controls were matched by gender, age (± 2 years), and ethnic group, with a predominance of middle-age European men. Participants in both groups had to be aged between 18 and 65 years and be fluent in Spanish to be included in the study. The exclusion criteria for participation were the following: having a history or current diagnosis of a psychiatric disorder based on the MINI interview, receiving pharmacological or psychological treatment, or suffering from severe medical disorders. Additionally, the long-term meditators were required to have practiced meditation continuously for more than 10 years before the start of the study (including the last 10 years) with a mean of at least 60 min/day of formal practice during the entire period. The type of meditation practiced is open-monitoring meditation (OM). During OM, the attentional scope is expanded, and the meditator remains attentive to any experience that might arise (perceptions, thoughts, emotional content, and/or subjective awareness), without selecting, over-identifying, judging, or focusing on

any particular object (Dahl et al. 2015; Lippelt et al. 2014). Information on participant's meditation experience was also assessed, including the number of months that the participants had practiced meditation over their lifetime.

The general characteristics of subjects included in the study are shown in Table 1. Gender, age, and ethnic group variables were matched to avoid significant differences between groups.

Procedure

From each subject, a peripheral blood sample was obtained by venous puncture in EDTA tubes. Blood samples were centrifuged at 4500 rpm for 15 min at 4 ° C. Buffy coat was preserved with RNAlater™ stabilization solution (Sigma-Aldrich,St Louis, MO) and stored at − 80 °C until further use. DNA was isolated by standardized methods (Miller et al. 1988). Next, genome-wide DNA methylation data was generated using Infinium HumanMethylation450 BeadChip array (Illumina, Inc., San Diego, CA, USA) (Sandoval et al. 2011), which was performed at the Roswell Park Cancer Institute Genomics Shared Resource (Buffalo, NY, USA). Briefly, 500 ng of genomic DNA from each PBL sample was bisulfate-treated and hybridized to the BeadChip according to the manufacturer's protocol. A total of 485,577 cytosine positions were interrogated throughout the human genome, covering the 99% of RefSeq genes and 96% of CpG islands. The percentage of methylation (β value) at each interrogated CpG site was calculated after normalization and quality control were performed.

In order to minimize the potential bias introduced by batch effects, we performed samples-to-batch allocation using the OSAT tool (Yan et al. 2012). Microarray image processing was carried out using the GenomeStudio methylation module (Illumina, Inc.). Background was corrected, and adjustment was performed to avoid type I/II assay chemistry bias. So as to minimize technical variation and improve data quality, we used the Dasen method (Pidsley et al. 2013) as a normalization tool.

Before performing differential methylation analysis, we removed probes overlapping common single nucleotide polymorphisms (SNPs) along with those probes classified as internal controls of the Illumina microarray. Additionally, probes

Table 1 Characteristics of study participants

	Range	Total sample (n = 34)	Controls (n = 17)	Meditators (n = 17)	p value
Age, Md (SD)		49.47 (8.16)	48.59 (9.91)	50.35 (6.11)	0.536
Sex, male		24 (70.60)	11 (64.70)	13 (76.50)	0.452
Education, university		18 (52.9%)	10 (58.8%)	8 (47.1%)	0.492
BMI, Md (SD)		24.32 (1.94)	23.73 (2.13)	24.91 (1.58)	0.078
Months of practice		106.47 (123.06)	0.00 (0.00)	212.94 (84.54)	< 0.001
MAAS, Md (SD)	1–6	3.93 (0.60)	3.42 (0.25)	4.43 (0.38)	< 0.001
FFMQ observing, Md (SD)	8–40	26.38 (2.76)	23.94 (1.03)	28.82 (1.43)	< 0.001
FFMQ describing, Md (SD)	8–40	27.44 (2.06)	26.00 (1.12)	28.88 (1.76)	< 0.001
FFMQ acting, Md (SD)	8–40	30.35 (2.89)	27.88 (1.50)	32.82 (1.43)	< 0.001
FFMQ non-judging, Md (SD)	8–40	29.29 (4.08)	26.41 (3.81)	32.18 (1.47)	< 0.001
FFMQ non-reacting, Md (SD)	7–35	24.00 (2.58)	22.88 (2.47)	25.12 (2.23)	0.009
HADS anxiety, Md (SD)	0–21	1.74 (1.52)	3.06 (0.90)	0.41 (0.51)	< 0.001
HADS depression, Md (SD)	0–21	2.50 (2.02)	4.12 (1.05)	0.88 (1.32)	< 0.001
CDRISC resilience, Md (SD)	0–100	27.91 (3.68)	24.53 (1.59)	31.29 (1.05)	< 0.001
SHS happiness, Md (SD)	4–28	23.77 (3.65)	21.00 (1.80)	26.53 (2.83)	< 0.001
SCS common humanity, Md (SD)	2–8	5.00 (1.16)	4.47 (1.01)	5.53 (1.07)	0.006
SCS mindfulness, Md (SD)	2–8	4.97 (1.53)	3.71 (0.77)	6.24 (0.90)	< 0.001
SCS self-kindness, Md (SD)	2–10	4.88 (0.91)	4.47 (0.88)	5.29 (0.77)	0.007
SLWS satisfaction, Md (SD)	5–35	28.15 (2.36)	26.71 (2.20)	29.59 (1.50)	< 0.001
AAQ-II avoidance, Md (SD)	7–49	18.65 (4.44)	22.41 (1.58)	14.88 (2.83)	< 0.001

Figures represent means, standard deviations (SD), and the p value associated with a t contrast between the control group and the meditator group, except for sex and education, where the figures represent frequencies and percentages (in parentheses) and the p value is associated with a χ^2 contrast. Range indicates the bounds of the scales for each psychological variable

located on the X and Y chromosomes were discarded along with probes that hybridized to multiple locations in the genome (Chen et al. 2013; Price et al. 2013). Probes that technically did not pass the Illumina quality threshold (1567 probes with bead count < 3 in > 5% of samples and 535 probes having 1% of samples with a detection p value > 0.05) were also removed. In the end, a total of 263,495 probes (representing CpG sites) were further analyzed for differential methylation (Supplemental Fig. S1). To identify major patterns of variation in methylation profiles, principal component analysis (PCA) of overall DNA methylation was applied to the dataset (Supplemental Fig. S2).

Some bisulfite sequencing validations of differentially methylated regions (DMRs) were performed. Genomic DNA (500 ng) was bisulfate-converted using the EpiTect bisulfite kit (QIAGEN, Redwood City, CA, USA) according to the manufacturer's instructions. Primer pair sequences were designed by MethPrimer (Li and Dahiya 2002) and are listed in Supplemental Table S3. PCR products were cloned using the TopoTA cloning system (Invitrogen, Carlsbad, CA, USA), and a minimum of 12 independent clones was sequenced for each examined subject and region. Methylation graphs were obtained by using QUMA software (Kumaki et al. 2008).

Measures

Methylation levels for each CpG and subject (β values/M values) were obtained from the array results. Participants also completed a battery of socio-demographics (age, sex, education, body mass index, total months of mindfulness practice) and health-related psychological questionnaires (Supplementary Material 1).

Data Analyses

Differential methylation analysis was performed by using several R /Bioconductor packages that compared methylation levels (β values/M values) between mindfulness meditators and controls. Our aim was to identify DMRs, defined as loci, containing concordant and significant changes for neighboring CpGs (two or more CpGs) (Jaffe et al. 2012). In order to increase the statistical power of the study, we applied recent methods that detect concordant and significant changes for neighboring CpGs to identify DMRs (Peters et al. 2015). Methylation differences were prioritized by the lowest p values to ensure the most consistent DMRs between MM and controls were included. These analyses identified sets of candidate loci with consistent differences in methylation in MM versus controls.

In addition, functional analysis in silico of DMRs was carried out. First, we performed a systematic, manual curation of the literature and databases (Genetic Association Database, CDC HuGE Published Literatur, and MalaCards database) to identify those genes related to diseases, including immune, neurological, and neuropsychiatric disorders along with other traits and biologically relevant functions among our set of DMRs.

In order to determine the biological significance of DMRs, gene-based enrichment analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID v.6.7; http://david.abcc.ncifcrf.gov) functional annotation tool (Subramanian et al. 2005). We report all gene ontology (GO) terms with a nominal p value less than 0.1.

Ingenuity Pathways Analysis (IPA) software from Ingenuity Systems® (Qiagen, Redwood City, California, USA) was used to expand the functional in silico analysis of the set of DMRs. IPA determined the most significantly enriched biological functions and/or related diseases by calculating the p value using Fisher's exact test. Analysis was performed to identify gene networks, canonical pathways, and functions enriched within our results. In addition, the "upstream regulator analysis" was also applied to predict upstream molecules potentially able to regulate the set of differentially methylated genes. The goal of this IPA tool is to identify upstream transcriptional regulators that may help to uncover new biological activities in our data.

To test whether DMRs were preferentially associated with specific transcription factor-binding motifs, we used the Hypergeometric Optimization of Motif Enrichment (HOMER) motif discovery tool (http://biowhat.ucsd.edu/homer/ngs/index.html). Searches for de novo motif were performed with the default settings: background selection matched on the percentage of guanine-cytosine (GC) content, the percentage of GC content normalization, oligo auto-normalization, regions for motif finding set as 200-bp windows centered on CpG sites. Motif enrichment (target versus background) was calculated using the cumulative binomial distribution.

Results

Socio-demographic and Psychological Features of Participants

Socio-demographic data were equally distributed between groups, but compared to controls, meditators showed a healthier profile in terms of psychological variables (Table 1).

Identification of Differentially Methylated Regions Between Mindfulness Meditators and Controls

We found 64 DMRs that passed our statistical threshold (β difference $\geq$ 0.05 and minimum p value $\leq$ 0.05), corresponding to 43 genes (Table 2). The mean length of

Table 2 Differentially methylated regions (DMRs) in PBL samples from long-term mindfulness meditators and controls analyzed by 450K Illumina BeadChip array

Genes	Chr	DMR-start	DMR-end	no.probes	Min. *p* value	beta.diff
NR4A2	chr2	157184401	157187204	13	4.15117E − 13	− 0.063
KBTBD11	chr8	1954777	1955196	4	3.90054E − 08	− 0.053
TYW3,CRYZ	chr1	75198403	75199117	8	8.36204E − 07	0.062
TRABD2B	chr1	48452465	48452740	3	1.29883E − 05	0.075
ZSCAN12L1	chr6	28058715	28058856	5	0.00012719	− 0.094
SERPINB9	chr6	2891973	2892050	2	0.00012719	0.062
MIR10B,HOXD4	chr2	177013630	177015044	12	0.000127669	− 0.056
UNCX	chr7	1266180	1267228	4	0.000393945	− 0.114
NA	chr11	67383377	67383802	6	0.000478885	− 0.092
C10orf11	chr10	77542302	77542585	7	0.000594424	− 0.059
WNK4	chr17	40934398	40934907	2	0.000632099	− 0.089
RPS6KA2	chr6	166876490	166877038	7	0.000731957	− 0.065
HTRA4	chr8	38831508	38832728	6	0.000798404	− 0.052
LZTS1	chr8	20112175	20112813	4	0.000944855	0.057
CLEC11A	chr19	51225848	51226849	7	0.001400841	− 0.058
MADCAM1	chr19	495355	495737	2	0.001456746	− 0.081
C8orf73	chr8	144654887	144655484	4	0.002101627	− 0.058
LAMA2	chr6	129251071	129251445	3	0.00210634	− 0.063
NA	chr12	114107712	114107765	2	0.00210634	0.071
MLPH	chr2	238406108	238406478	3	0.003007912	− 0.056
RTKN	chr2	74668976	74669387	4	0.003786336	− 0.072
ADAM32	chr8	38964500	38965492	10	0.004423946	− 0.053
SRXN1	chr20	633418	634604	4	0.004924204	− 0.062
ZNF385D	chr3	22412385	22413680	5	0.005558229	− 0.103
TACR3	chr4	104641319	104641548	2	0.007012852	− 0.069
EVPLL	chr17	18286412	18286644	2	0.007191908	0.052
TRPC3	chr4	122853464	122854336	6	0.007613165	0.054
NR4A2	chr2	157183013	157183291	2	0.008188896	− 0.076
MYL5,MFSD7	chr4	675137	675827	3	0.008481849	0.126
NA	chr1	149144820	149145243	5	0.008751084	− 0.118
NA	chr1	149162430	149162518	3	0.008912857	− 0.083
NA	chr4	1514317	1514621	2	0.008962804	− 0.086
NA	chr3	14615444	14615956	4	0.009026339	0.060
RPS6KA2	chr6	167070053	167070616	3	0.009173941	0.081
HIST1H3D	chr6	26195488	26195995	4	0.009726208	0.068
APOB	chr2	21267113	21267334	4	0.010026859	− 0.051
DNAH7	chr2	196933546	196933852	6	0.010215384	0.071
NA	chr8	101348456	101348501	3	0.011525051	− 0.051
C19orf60	chr19	18698825	18699118	2	0.012018869	− 0.066
WNK4	chr17	40936078	40937270	7	0.012659477	− 0.059
ODZ4	chr11	78614359	78614522	2	0.013522189	0.076
LHX8	chr1	75590483	75591353	5	0.014181112	− 0.108
PACRG,PARK2	chr6	163149167	163149674	5	0.014516207	− 0.055
NCOR2	chr12	125002007	125002474	3	0.016301777	− 0.059
PRSS21	chr16	2867773	2868001	2	0.021467194	0.064
GPR31	chr6	167571172	167571803	5	0.023259942	− 0.071
ZNF385D	chr3	21792248	21792991	8	0.023373579	0.053
NAV1	chr1	201617847	201618209	4	0.027339757	− 0.051
NA	chr5	154026371	154026979	4	0.02840663	− 0.076

Table 2 (continued)

Genes	Chr	DMR-start	DMR-end	no.probes	Min. *p* value	beta.diff
NA	chr16	51671357	51671563	2	0.028596197	− 0.066
LOC150197	chr22	20192371	20192485	2	0.028596197	0.074
PTCH1	chr9	98279753	98280076	2	0.028716062	− 0.051
CTSZ, TUBB1	chr20	57582706	57583076	7	0.02952523	− 0.051
RUFY1	chr5	178986131	178986728	7	0.029542821	− 0.063
HRH1	chr3	11267020	11267525	4	0.031293469	− 0.061
GSTA4	chr6	52858998	52859107	3	0.033941429	− 0.051
VHL	chr3	10184319	10184584	2	0.036129484	0.075
ERICH1-AS1	chr8	709576	709692	2	0.036605753	− 0.059
PRR25	chr16	857454	857863	3	0.036715428	0.106
LOC149837	chr20	5485245	5485294	5	0.037797935	− 0.090
C13orf45	chr13	76444798	76444831	3	0.039364018	− 0.051
NA	chr6	28446794	28447107	4	0.041039298	− 0.071
APOC2	chr19	45449297	45449301	2	0.041719648	0.062
NA	chr10	102381293	102381344	2	0.046623988	− 0.082

The table shows 64 DMRs prioritized by the lowest *p* value criteria

PBL peripheral blood leukocytes, *NA* not applicable, *Chr* Chromosome, *DMR-start* coordinates of the beginning of each DMR annotated by the UCSC hg19 build, *DMR-end* coordinates of the end of each DMR annotated by the UCSC hg19 build, *No. probes* total number of differentially methylated probes within each DMR, *beta.diff* difference in average methylation between mindfulness practitioners and controls for each DMR

mindfulness-related DMRs was 499.6 bp. Mindfulness-related DMRs encompassed an average of 4.3 differentially methylated CpG sites per region, and 3 DMRs contained 10 or more differentially methylated CpG sites, corresponding to *NR4A2*, *HOXD4*, and *ADAM32* genes, respectively (Table 2). Most of the DMRs, specifically 45 DMRs (70.3%), were hypomethylated in MM while only 19 DMRs (29.7%) were hypermethylated in MM compared to control subjects (Fig. 1). Differential methylation between MM and control subjects showed a mild to moderate effect size, since the average absolute β difference was 0.070 for the 64 DMRs related to mindfulness practice (Fig. 1). The maximum absolute β difference (0.126) was observed for a CpG island overlapping the 3′untranslated region (3′UTR) of *MYL5* and *MFSD7* genes within the subtelomeric region of the short arm of chromosome 4 (Supplemental Fig. S3).

Of note, DMRs were not randomly distributed throughout the genome; instead, they tended to cluster at specific hot spots, as it is shown by the CIRCOS plot (Fig. 2). Up to 9 out of 64 DMRs were placed within telomere-proximal regions of chromosomes 4, 5, 7, 8, 16, 19, and 20. Moreover, additional six DMRs were located near the subtelomeric region of chromosomes 2, 6, and 8. On the whole, 23.4% of mindfulness-related DMRs were found nearby telomere regions (Fig. 3).

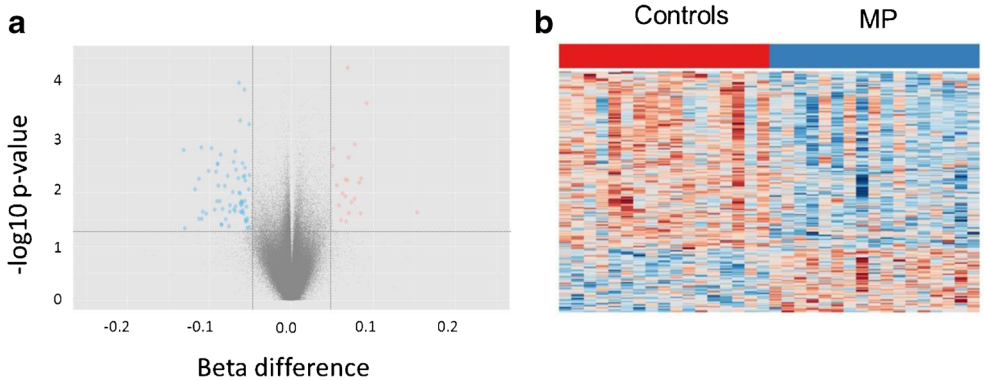

Fig. 1 **a** The volcano plot shows the results of differentially methylated CpGs between long-term meditators and controls based on beta difference versus statistical significance. Red dots correspond to hypermethylated CpGs while blue dots correspond to hypomethylated CpGs. Gray dots represent CpGs that did not pass the statistical thresholds. **b** Heat map of methylation values (beta values) shows differentially methylated CpGs in meditation practitioners (MPs) and controls. Increase in methylation levels is shown as red boxes, while decrease in methylation is shown as blue boxes. Columns correspond to subjects, and lines correspond to CpGs. Hypomethylated CpGs are more abundant within the MP group than in the control group (Color figure online)

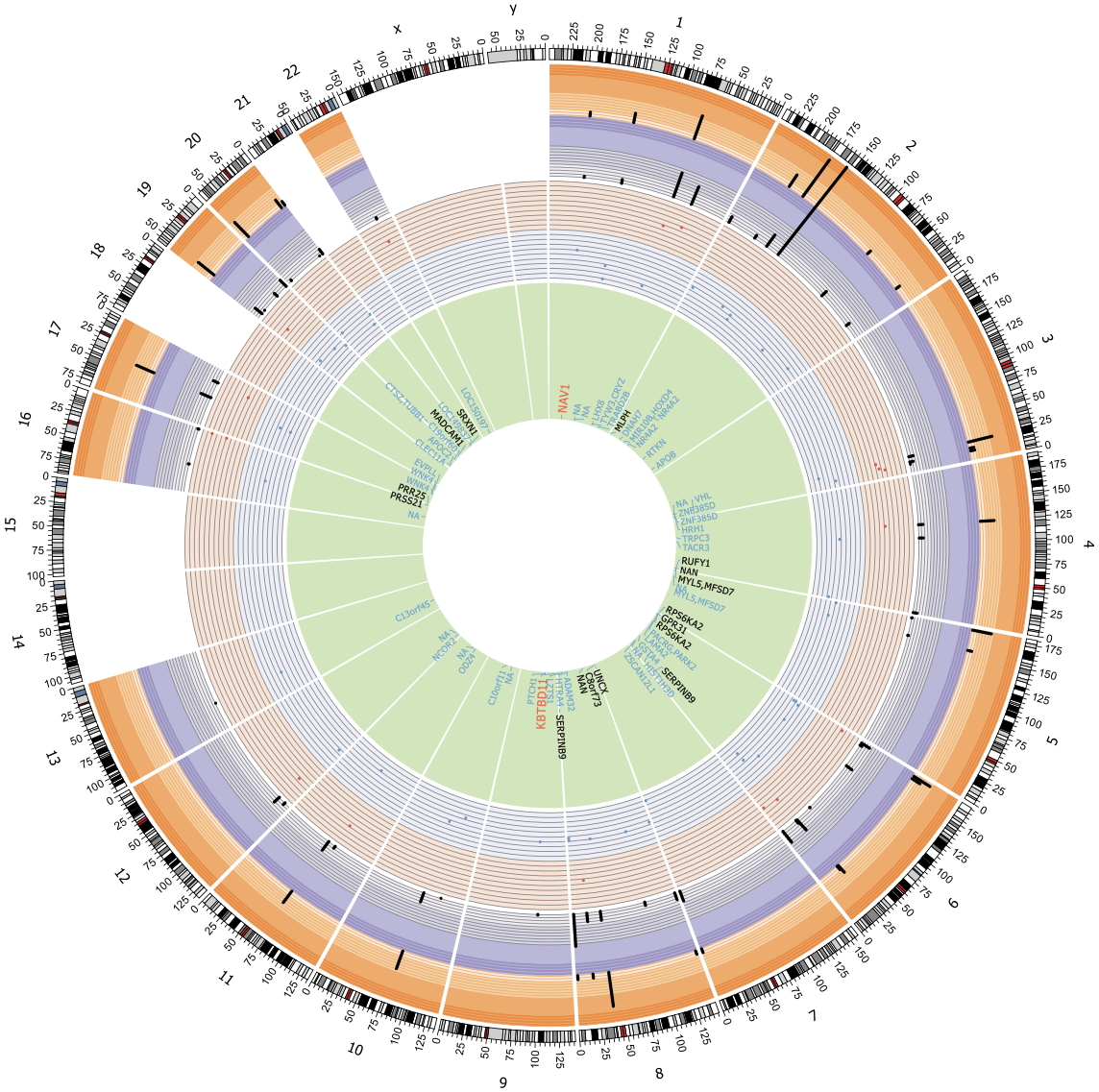

Fig. 2 The CIRCOS plot shows differentially methylated regions (DMRs) between meditation practitioners and controls across the autosomes. X and Y chromosomes were excluded from the analysis. Only those chromosomes harboring differentially methylated regions are represented in the colored circles. The perimeter of the circular figure represents the human chromosomes, showing the cytogenetic bands and centromeres (in red). The orange circle represents the minimum *p* value for probes in a particular DMR, while the violet circle shows the mean *p* value for all the probes in a particular DMR. Red and blue circles represent the results of the differential analysis with each of the DMRs, including gains in methylation (red dots) and losses in methylation (blue dots). The inner green circle reports the names of the genes associated to each of the DMRs. In red font, those genes that passed the genome-wide significance threshold are shown. In black font, those genes located within telomere regions (Color figure online)

In addition, two DMRs showed *p* values below genome-wide significance threshold (nominal *p* value < 5E − 08). One of these DMRs was located within the *NR4A2* gene and showed up to 13 differentially methylated CpGs spanning the 5′UTR region and the gene body (absolute β difference = 0.063; *p* value = 4.15E − 13) (Table 2, Fig. 4). This region was successfully validated by bisulfite cloning sequencing confirming that the *NR4A2* gene is hypomethylated in MM peripheral leukocytes (Fig. 4). The second DMR with *p* value below the genome-wide significance threshold was placed at the 3′ UTR of the *KBTBD11* gene and contained four differentially methylated CpGs (absolute β difference = 0.053; *p* value = 4.90E − 08) (Table 2, Supplemental Fig. S4).

Mindfulness-Related DMRs Are Preferentially Associated with Brain and Cardiovascular Human Diseases

We also wanted to know if these 64 DMRs were preferentially related to specific human diseases or traits, so we performed a systematic manual curation of the literature and databases to assess this particular question (see the "Methods" section). Out of the 64 DMRs, 31 DMRs (48.4%) showed association

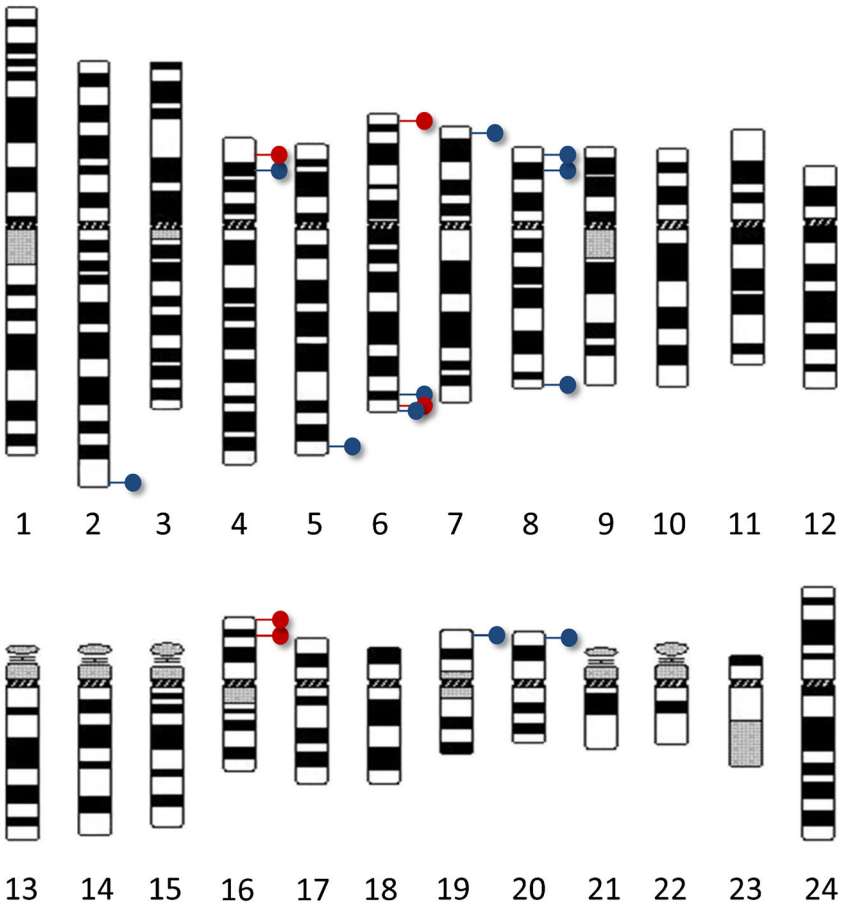

Fig. 3 DMRs located in subtelomeric loci. The graph shows a human ideogram with the mindfulness-related DMRs that are located within or nearby telomeric regions. In red, gain in methylation DMRs, and in blue, loss in methylation (Color figure online)

with diseases and human traits (Supplemental Table S1), some of these DMRs overlapping several diseases or traits.

We found 16 DMRs (25%) located in genes associated with psychiatric (bipolar disorder, schizophrenia, autism, major depressive disorder, and attention deficit disorder) or neurological conditions, including 9 DMRs (14%) in genes related to neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis). In addition, 15 DMRs (23.4%) were placed in genes related to vascular risk factors, such as hypertension, diabetes mellitus, and lipid levels or genes directly associated with cardiovascular disease, such as stroke and coronary or peripheral artery disease. Moreover, nine DMRs (14%) were related to cancer, such as leukemia and colorectal, prostate, and breast cancers. Finally, we only found five DMRs (7.8%) in genes associated with immune diseases, such as multiple sclerosis, inflammatory bowel disease, atopic dermatitis, or sclerosing cholangitis.

Functional Analysis In Silico of Mindfulness-Related DMRs

We further asked whether the set of mindfulness-related DMRs was enriched for genes involved in specific functions, so we performed GO annotation using the DAVID tool. In the biological process category, we found that GO terms most strongly enriched in our set of 64 DMRs included cellular response to unfolded protein, regulation of dopamine, protein catabolic processes, or regulation of synaptic plasticity, among others (Table 3). Regarding the cellular component category, the GO terms enriched involved a set of different lipoprotein particles, while the most strongly associated GO term within the molecular function category was the phospholipase binding (Table 3).

Next, we used the IPA tool to better characterize our set of DMRs and seek for new relationships. The analysis confirmed that our set of mindfulness-related DMRs was enriched in genes with a functional role in neurological and psychiatric disorders, cardiovascular diseases, and cancer (p value range = 4.82E − 02–3.67E − 04) (Supplemental Fig. S5). Lipid metabolism and related functions were highly enriched in our set of differentially methylated genes (p value = 3.67E − 04) (Fig. 5), *APOB*, *APOC2, HRH1, PTCH1, CLEC11A* and *NCOR* being the main genes involved in these functions. Consistent with this result, the top canonical pathways overrepresented in our set of differential genes were LXR/RXR and FXR/RXR, which are crucial to regulate cholesterol, fatty acid, and glucose homeostasis, along with the atherosclerosis signaling pathway (Supplemental Fig. S6).

In addition, to gain insight into upstream regulators of the 43 mindfulness-related differentially methylated genes, we used the Upstream Analysis tool from the IPA software. Eight predicted upstream regulators were identified prioritized

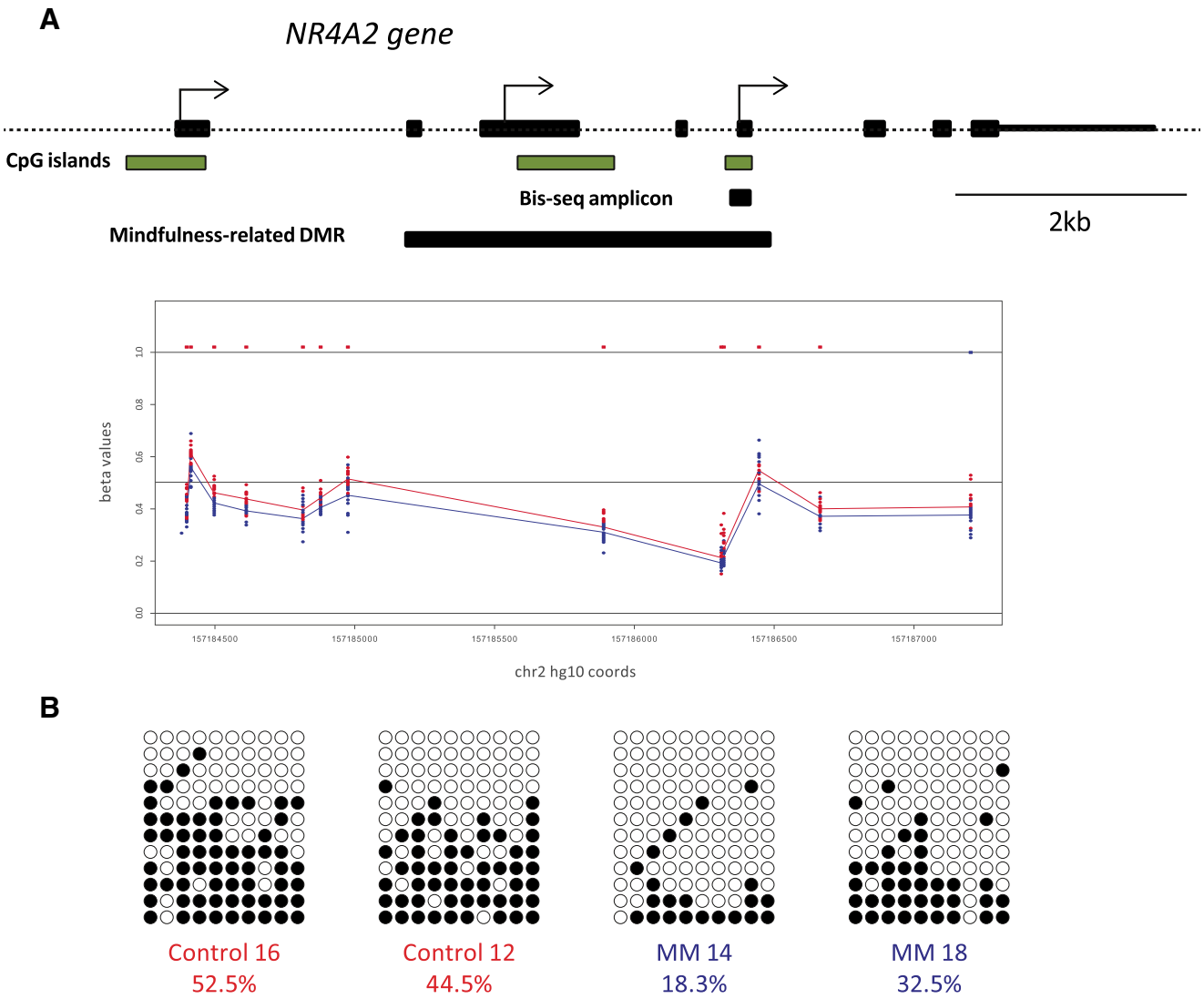

Fig. 4 DNA methylation levels within the *NR4A2* gene are decreased in peripheral blood leukocytes (PBLs) from meditators compared to controls. **a** The upper graph shows the genomic map of *NR4A2* and the position of the mindfulness-related DMR that overlaps this gene. Arrows denote transcription start sites for different transcripts. Black boxes represent exons; green boxes correspond to CpG islands. The amplicon designed to perform bisulfite sequencing cloning is also shown. The graph below shows the 450K methylation values (beta values) for controls (red) and meditators (blue) across the DMR partially overlapping the *NR4A2* gene. **B.** The picture shows the result of validation of the *NR4A2* DMR by bisulfite cloning sequencing (Bis-seq). The methylation pattern at the CpG site resolution for the Bis-seq amplicon within the *NR4A2* gene is shown in four different samples. Black and white circles represent methylated and unmethylated cytosines, respectively. Each column symbolizes a unique CpG site in the examined amplicon, and each line represents an individual DNA clone. Global percentage of methylation for each analyzed sample (control or meditator) at this particular amplicon is indicated at the bottom of each sample (Color figure online)

by *p* value, with the cytokine TNF showing the highest number of relationships (Fig. 5). None of them had been previously linked to mindfulness with the exception of TNF (Elsenbruch et al. 2005; Rosenkranz et al. 2013) which encodes tumor necrosis factor alpha, a cytokine involved in a wide range of human diseases, including neurological, psychiatric, cardiovascular, cancer, and immune disorders. Supporting the relevance of TNF as a crucial regulator of the mindfulness-related genes, the main network associated to our set of data (focused molecules 14, score 30) illustrates the central role of TNF in the set of mindfulness-related DMRs (Supplemental Fig. S7). Moreover, the second network related to our set of DMRs (focused molecules 10, score 19) reveals the central role of NF-κB signaling in the epigenetic response to mindfulness practice (Supplemental Fig. S8).

Finally, we found by using the HOMER tool that DMRs were enriched in TFB (transcription factor-binding) motifs for transcription factors such as Meis3 or Mafk, both linked to the survival of pancreatic beta cells and insulin metabolism. Other functions overrepresented in the TFB motifs related to the DMRs were neural and heart development, oxidative stress and hypoxia, proliferation and differentiation of hematopoietic progenitor cells, immune Th2-helper response, and DNA repair (Supplemental Table S2).

Table 3 Gene ontology (GO) enrichment analysis

GO category	GO term	Description	Percent of genes found	Fold enrichment	*p* value
Biological process	34620	Cellular response to unfolded protein	4.167	190.761	0.010
Biological process	42053	Regulation of dopamine metabolic process	6.250	114.457	0.000
Biological process	44257	Cellular protein catabolic process	4.167	44.885	0.043
Biological process	33344	Cholesterol efflux	4.167	29.348	0.065
Biological process	48167	Regulation of synaptic plasticity	4.167	24.614	0.076
Biological process	42157	Lipoprotein metabolic process	4.167	19.565	0.095
Biological process	32355	Response to estradiol	6.250	9.230	0.040
Biological process	10628	Positive regulation of gene expression	8.333	5.938	0.028
Biological process	6508	Proteolysis	12.500	4.749	0.007
Cellular component	34363	Intermediate-density lipoprotein particle	4.167	216.690	0.009
Cellular component	42627	Chylomicron	4.167	61.912	0.031
Cellular component	34362	Low-density lipoprotein particle	4.167	61.912	0.031
Cellular component	34361	Very-low-density lipoprotein particle	4.167	43.338	0.044
Molecular function	43274	Phospholipase binding	4.167	43.156	0.044
Molecular function	30544	Hsp70 protein binding	4.167	25.058	0.075
Molecular function	8270	Zinc ion binding	14.583	2.279	0.076
Molecular function	31072	Heat-shock protein binding	4.167	18.947	0.098

GO gene ontology, *Hsp* heat-shock protein

Discussion

We showed that a gene-specific epigenetic response to mindfulness practice occurs in peripheral blood leukocytes in long-term meditators. We also highlighted underlying biological processes that might be influenced by mindfulness through changes in DNA methylation patterns.

The beneficial effects of mindfulness on human health and disease have been documented, but the mechanisms underlying these effects are yet not well-understood. Our study suggested that epigenetic modifications, in particular DNA methylation changes in a set of specific genes, could contribute in part to explain these effects. DNA methylation is one of the epigenetic mechanisms regulating gene expression under environmental and behavioral influences. Mindfulness-related DNA methylation changes may involve alterations of specific transcriptional networks in long-term meditators. Indeed, it has been previously shown that yoga and mindfulness practice reduce the expression of pro-inflammatory genes in blood cells (Creswell et al. 2012; Kaliman et al. 2014; Ravnik-Glavač et al. 2012; Saatcioglu 2013), including reduced pro-inflammatory nuclear factor NF-κB signaling and increased activity of interferon response factors (IRF), which are molecular patterns previously linked to stress (Black et al. 2013). Despite these observations on gene transcriptional changes, no data regarding DNA methylation patterns in response to long-term mindfulness practice has been previously described to our knowledge. We have identified a set of 64 genomic regions, corresponding to 43

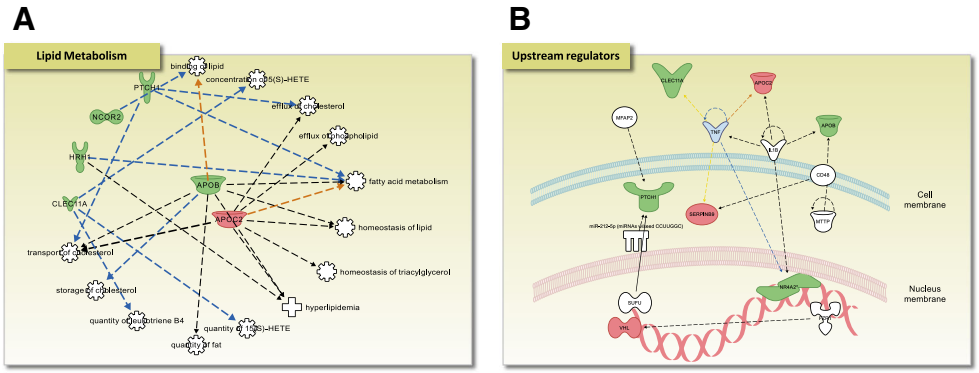

Fig. 5 IPA analysis of mindfulness-related differentially methylated genes. **a** The graph shows functions and diseases related to lipid metabolism which are enriched in our results. **b** The diagram shows the eight upstream regulators of our set of differentially methylated genes (in white) along with the predicted regulated genes (red upregulated, green downregulated) distributed across cell compartments. TNF was the molecules with the highest number of relationships and is represented in pale blue (Color figure online)

genes, which showed differential methylation in long-term meditators compared to controls.

These epigenetic modifications were not limited to individual CpG sites. On the contrary, they have been found to involve several CpG sites (array probes) in confined genomic regions with an average of 4.3 differentially methylated CpGs per region. In addition, our bisulfite cloning sequencing validation experiments proved that differential methylation affects multiple contiguous CpGs, as it was shown in the case of *NR4A2* and *KBTBD11* genes. Most of the DMRs were hypomethylated in long-term meditators compared to controls. Notably, the abundance of hypomethylated regions in the differential analysis contrasts with results from other Infinium 450K methylation arrays performed in pathologic conditions (De De Jager et al. 2014; Graves et al. 2014; Jo et al. 2016; Masliah et al. 2013; Rhead et al. 2017; Urdinguio et al. 2015), where a predominance of gene-specific hypermethylation is usually observed.

Among those genes showing differential methylation between long-term mindfulness practitioners and controls, we found two hits that passed the genome-wide significance threshold. A DMR containing up to 13 differentially methylated CpGs overlaps the *NR4A2* gene, which encodes a nuclear transcriptional regulator that is crucial to neuronal development, particularly to the maintenance of the dopaminergic system (Jankovic et al. 2005). *NR4A2* has shown a protective effect in neurons against apoptotic stress (Sleiman et al. 2009), and mutations in this gene have been associated with disorders related to dopaminergic dysfunction, including Parkinson disease, schizophrenia, and manic depression (Buervenich et al. 2000; Liu et al. 2015; Sleiman et al. 2009). Most recently, the orphan nuclear receptor 4A proteins (NR4A), including *NR4A2*, have been reported to modulate regulatory T cells (Treg), which in turn are crucial for maintaining immune homeostasis, pointing to a therapeutic potential of NR4A proteins in treating immune disorders (Won and Hwang 2016). Indeed, *NR4A2* provides protection in a murine model of multiple sclerosis, by promoting an increase in Treg and limiting effector T cells (Saini et al. 2016). The second gene, *KBTBD11*, encodes a protein highly expressed in the brain according to data obtained from the Genotype-Tissue Expression (GTEx) portal (Melé et al. 2015) and was also related to Parkinson's disease in a genome-wide association study (Fung et al. 2006). A nearby region to the *KBTBD11* gene has been reported to be a strong haplotype-related allele-specific DMR in T cells (Do et al. 2016). The latter region does not overlap our mindfulness-related DMR, but both results point to the potential significance that differential methylation within the *KBTBD11* genomic region may have in human health.

Mindfulness-related DMRs were not randomly distributed throughout the genome, since up to 23.4% of mindfulness-related DMRs were found to be placed close to telomere regions. This result added evidence to the idea that mindfulness practice is somehow related to telomere biology. Accordingly, our group previously showed a positive relationship between meditation practice and telomere length (Alda et al. 2016) in long-term meditators. Nevertheless, further studies are necessary to better understand this intriguing association.

An interesting result of our study was that almost half of the mindfulness-related DMRs (48.4%) were directly linked to common human diseases, brain and cardiovascular disorders being the most common associations, followed by cancer and immune conditions. Among brain diseases, neurodegenerative disorders seemed to be particularly associated to the mindfulness-related DMRs, including Alzheimer's and Parkinson's diseases. This is in accordance with previous reports showing larger hippocampal dimensions in meditation practitioners (Luders et al. 2013) and structural brain changes in patients with Parkinson's disease who received a mindfulness-based intervention (Pickut et al. 2013). Our study supports the idea that long-term mindfulness practice might affect human health and pathogenic processes through, at least in part, distinct epigenetic mechanisms. Even though a stable DNA modification, DNA methylation is potentially modifiable and may underlie some of the transcriptional or structural changes that are observed in meditation practitioners.

DNA methylation changes affected sets of genes that are related to specific biological, cellular, and molecular functions. In particular, lipid metabolism emerged as a consistent biological process that could drive some of the mindfulness effects, since cholesterol and lipoprotein transport along with several lipoprotein particles were found in our functional in silico analysis and were the most enriched functions in the IPA analysis. This is in agreement with the fact that mindfulness-related DMRs are highly associated with cardiovascular diseases, since lipid metabolism alterations are well-known risk factors for cardiovascular events. In addition, altered lipid metabolism has also been related to brain disorders, such as Alzheimer's disease (Reitz 2013). Notably, our results highlighted the relevance of TNF and NF-κB signaling in the epigenetic response to mindfulness practice. Indeed, previous results of biomarkers and gene expression studies support this notion. For instance, TNF was reduced in the saliva of women with depressive symptomatology after mindfulness training (Walsh et al. 2016). Likewise, mindfulness practice reduced pro-inflammatory nuclear factor NF-κB signaling in blood cells (Black et al. 2013), as we mentioned above. Indeed, both molecules were intrinsically associated since TNF is a potent inductor of the NF-κB signaling pathway. This is most significant, since dysregulation of NF-κB signaling pathway mediates the pathogenesis of multiple human diseases and, therefore, has become a major therapeutic target (Panday et al. 2016).

Finally, as a number of transcription factors (TFs) seemed to bind their sequence motifs in a methylation-sensitive manner (Domcke et al. 2015) in the genome, we explored which

TFs may bind within or nearby the mindfulness-related DMRs and, therefore, be affected by changes in DNA methylation. By using the HOMER tool, we observed that mindfulness-related DMRs were enriched in motifs for certain TFs such as Meis3 and Marf (Supplemental Table S2), which are both related to insulin metabolism and survival of pancreatic beta cells. Most of the motifs were for TFs that show a link with neural development or neuroprotection and cardiac development. Among these TFs emerges Nrf2, which plays a key role in the response to oxidative stress and has anti-inflammatory and neuroprotective properties. The strongest enriched motifs correspond to MZF1 and HBP1 TFs, which are involved in cardiogenesis, hematopoiesis, and neuronal differentiation along with cell senescence and tumorigenesis. On the whole, mindfulness-related DMRs seem to localize close to regulatory regions for certain biological and molecular processes consistent with their disease pathological associations.

In conclusion, our results suggest that there is a consistent association of long-term meditation practice with a predominant loss of CpG methylation in distinct genomic regions. Biological processes related to lipid or insulin metabolism may be affected by mindfulness through epigenetic modifications. At the molecular level, our functional analysis suggests a crucial role of TNF and NF-κB signaling pathway in the response to mindfulness practice. Moreover, methylation changes tend to occur in genomic regions where TFs related to cardiac and neural development, response to oxidative stress and inflammation, tumorigenesis, and immune response are predicted to bind. Our study supports the notion that there may be some epigenetic changes in the interplay between meditation and a specific set of genes that might mediate the effects on particular health conditions, including neurological and psychiatric disorders, cardiovascular diseases, and cancer. The present study begins to shed some light on the mechanisms underlying the effects of long-term mindfulness practice on human health and disease at the molecular level, such as the potential involvement of TNF and NF-κB signaling pathway. Further studies in other groups of meditators, particularly in short-term meditation interventions, are warranted to test whether there is a dose-response effect of meditation on these methylation changes.

Limitations

The main limitation of the present work was related to the design used, a retrospective case-control study, which makes it difficult to establish clear lines of causality. This problem is not easy to tackle, unless we use panel studies with medium- or long-term longitudinal data. However, the comparison groups that were used showed significant differences in health-related psychological outcomes, favoring the meditation group, which is expected and is probably due to long-term meditative practice. This does not completely save the previously referred limitation but permits sustaining causality at least from a heuristic or hypothetical point of view. The reduced size and relative homogeneity of the sample, combined with the multiple testing procedures, were also limitations which could lead to find spurious findings, but the used approach analyzed differences in regions in which changes in CpGs went in the same direction, rather than only individual CpGs, reducing the possibility of false positives (Peters et al. 2015). Another limitation may arise from the fact that 450K Illumina arrays do not cover all the CpGs in the genome, in contrast to other technologies such as whole-genome bisulfite sequencing. Therefore, methylation modifications at different genomic loci not included in the array may be missed. Nevertheless, the arrays are designed to cover the 99% of RefSeq genes and 96% of CpG islands so most parts of the cis-regulatory regions are covered by the assay. They also provide base pair-resolution DNA methylation measurements, and what is more important is they have been successfully used to reveal interesting regulatory regions and biological pathways in a consistent manner for the last decade.

Acknowledgments We are very grateful to the subjects that generously donated their samples to our study.

Author Contributions JGC contributed to the study design, obtaining of funding, acquisition of data, analysis and interpretation of data, supervising of the study, and drafting/revising of the manuscript for content. MPG contributed to the acquisition of data, analysis and interpretation of data, and drafting/revising of the manuscript for content. AL contributed to the analysis and interpretation of data, statistical analysis, figure design, and drafting/revising the manuscript for content. AU contributed to the analysis and interpretation of data, bisulfite experiments, figure design, and drafting/revising of the manuscript for content. MR contributed to bisulfite experimental work, figure design, and drafting/revising of the manuscript for content. XMM contributed to the analysis and interpretation of data, figure design, and drafting/revising of the manuscript for content. LP contributed to the acquisition of data, experimental work, and drafting/revising of the manuscript for content. AP contributed to the analysis and interpretation of data and drafting/revising of the manuscript for content. JMM contributed to the analysis and interpretation of data and drafting/revising of the manuscript for content. MM contributed to the study design, obtaining of funding, acquisition of epigenomic data, analysis and interpretation of data, statistical analysis, supervising of the study, and drafting/revising of the manuscript for content.

Funding Information The project has received funding from the Network for Prevention and Health Promotion in Primary Care (RD12/0005/0006) grant from the Instituto de Salud Carlos III of the Ministry of Economy and Competitiveness (Spain), co-financed with European Union ERDF funds (FEDER "Una manera de hacer Europa"). The funding source did not have any influence on the design of the study, data collection and analysis, or writing of the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval All procedures performed in this study (number PI13/0056) involving human participants were in accordance with the ethical standards of the Aragon Ethics Regional Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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