Original Experimental

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DNA methylation changes in genes involved in inflammation and depression in fibromyalgia: a pilot study

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Abstract

Objectives: The present pilot study aims to investigate DNA methylation changes of genes related to fibromyalgia (FM) development and its main comorbid symptoms, including sleep impairment, inflammation, depression and other psychiatric disorders. Epigenetic modifications might trigger or perpetuate complex interplay between pain transduction/transmission, central pain processing and experienced stressors in vulnerable individuals.

Methods: We conducted DNA methylation analysis by targeted bisulfite NGS sequencing testing differential methylation in 112 genomic regions from leukocytes of eight women with FM and their eight healthy sisters as controls.

Results: Tests for differentially methylated regions and cytosines brought focus on the *GRM2* gene, encoding the metabotropic glutamate receptor2. The slightly increased DNA methylation observed in the *GRM2* region of FM patients may confirm the involvement of the glutamate pathway in this pathological condition. Logistic regression highlighted the simultaneous association of methylation levels of depression and inflammation-related genes with FM.

Conclusions: Altogether, the results evidence the glutamate pathway involvement in FM and support the idea that a combination of methylated and unmethylated genes could represent a risk factor to FM or its consequence, more than single genes. Further studies on the identified biomarkers could contribute to unravel the causative underlying FM mechanisms, giving reliable directions to research, improving the diagnosis and effective therapies.

Keywords: biomarkers; DNA methylation; epigenetics; fibromyalgia.

Introduction

Fibromyalgia (FM) is a pathological condition characterized by abnormal pain processing leading to chronic widespread pain (CWP) [1–4]. Because of the extensive array of symptoms associated with the condition, including disrupted sleep, depression, mental health disorders and low grade inflammation, a long debate on its definition and classification criteria [5–8] has made its diagnosis complex [9]. Recently, the worldwide prevalence of FM has been reported to range from 1 to 5% of the general population [10], being more common in females [11, 12]. Since the disease leads to a poor quality of life and high medical costs often without positive outcome of the selected treatment, FM represents a major social and health problem.

The etiopathogenesis of this multifactorial condition and the role of comorbidities, if causes or symptoms of the disease, are still a matter of debate. One of the hypothesized

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mechanisms in the FM pathophysiology is central sensitization [13], in which nociceptors, neurons and glia processing pain signals seem to undergo alterations and become sensitized in vulnerable individuals [14]. Thus, it is proposed that vulnerability to pain in patients with FM may reflect differences in central mechanisms that have been shown determined by both genetic and environmental factors.

The genetic influence was supported by a high prevalence of FM and reduced pressure pain thresholds among the offspring of FM mothers [15, 16]. Single-nucleotide polymorphisms affecting fiber connectivity and cognition [17] and related to the serotonergic [18], dopaminergic, catecholaminergic [19–21] and the endogenous opioid system [22–24] have been investigated using candidate gene approaches. However many of these studies failed to provide association with FM or related symptoms [25]. Then genome wide scans with no a priori assumptions tried to override confounding factors arising from FM comorbidities, highlighting a probable inflammatory basis of the syndrome [26] and a potential role of central nervous system (CNS) dysfunction [27].

Environmental influences have also been shown to play a great role in FM, especially daily life personal stressful experience, childhood maltreatment, such as neglect, emotional abuse and traumatic experiences. A complex interaction of genetic factors with dysfunctions in the HPA (hypothalamic-pituitary-adrenal) axis has been hypothesized to determine an individual's predisposition to somatic or psychological pathologic response to trauma [28].

To date, the above identified genetic and environmental factors have not fully explained the etiology of FM [29]. The molecular mechanisms responsible for the increased pain sensitivity in response to external stimuli could be DNA sequence–independent mechanisms regulating gene expression [30]. Evidence is emerging for a central regulatory role of epigenetics in influencing the neurobiological mechanisms of chronic pain generation [31].

Among the epigenetic changes, DNA methylation, the addition of a methyl group on the fifth positions of the cytosine in the DNA, catalyzed by DNA methyltransferases, stably alters gene expression in response to transient stimuli [32], potentially reflecting and revealing environmental predispositions to FM. This epigenetic change occurs mainly in the context of CG dinucleotides, which tend to cluster in regions called CpG islands with a GC content of at least 50%. Approximately 60% of gene promoters are associated with CpG islands [33].

A few studies have investigated the methylome in blood cells of FM subjects [34]. Menzies and coworkers (2017) found differentially methylated sites located in genes belonging to biological clusters significantly related to neuron differentiation, development and chromatin compaction [35]. In addition, a hypomethylated DNA pattern in FM patients compared to controls was found enriched in genes implicated in stress response and DNA repair/free radical clearance [36]. Another study investigated the DNA methylation status in CWP conditions, identifying differentially methylated CpGs in malate dehydrogenase 2 (*MDH2*; p-Value 0.017), tetranectin (*CLEC3B*; p-Value 0.039), and heat shock protein beta-6 (*HSPB6*; p-Value 0.016) [37]. An epigenome-wide methylation scan through MeDIPseq in whole blood DNA from 1708 monozygotic and dizygotic Caucasian twins highlighted neurological pathways' involvement in CWP, with association signals mapping in or near to *IL17A*, *ADIPOR2*, and *TNFRSF13B* [38].

Based on this promising evidence and the technology advances in epigenetic analyses, the present pilot study aimed to investigate changes in DNA methylation potentially affecting the genes related to FM development and its main symptoms by comparing FM women with their healthy sisters. To this purpose, we measured the presence of this epigenetic mark through a target enrichment-designed library specifically including promoters and CpG regions of genes previously found associated with FM, CWP, depression and other psychiatric disorders, sleep problems and inflammation. The current pilot study will guide further analyses for replicating preliminary results in a larger sample.

Methods

Subjects

A subset of 16 participants (8 FM women patients and eight related healthy sisters) were selected for the present study from a large sample of 543 families in which at least one member was diagnosed with FM. The FM patients were recruited according to the following criteria: subjects with FM diagnosis assessed by a professional specialist in rheumatology (Hospital of Pontevedra, Spain) and by the Unit of Pain of the CHUS (Complejo Hospitalario Universitario de Santiago, Spain) using the ACR (American College of Rheumatology) 2010 criteria. All the participants were classified into the diagnostic groups described in Table S1. The eight FM women were randomly selected among those belonging to diagnostic group 5 (FM patients, with and without comorbid symptoms/disorders related to the syndrome, but no other pathologies) and having at least one healthy sister with no pain (group 1). All the experiments were performed in accordance with the relevant guidelines and regulations and the study design was approved by the Ethics Committee of Galicia, Spain (Registration Code: 2013/582; Amendment: November 2017), and written informed consent was obtained from all participants. All the subjects accepted to enter the study as volunteers.

Demographic and clinical assessment

The eight (8) unrelated Caucasian FM patients were females, aged 39–63 years (mean age 51 ± 7.87 years). For each FM patient, a healthy sister, a total of eight (8) subjects (females, aged 33–71years, mean age 52.6 ± 12.3 years), was selected as control.

All the participants, subjects and controls, were submitted to a clinical interview about demographic data and the following tests were administered (Table S2): Fibromyalgia Impact Questionnaire (FIQ) [39, 40]; Visual Analog Scales (VAS) to assess the core symptoms of FM [41]; Pittsburgh Sleep Quality Inventory (PSQI) [42, 43]; Beck Depression Inventory (BDI) [44, 45].

Exclusion criteria

Exclusion criteria were the presence of other chronic pain diseases or other disorders that may explain the main symptoms of FM. For healthy controls, the requirement was no acute or chronic pain problems, or mental disorders.

Sample collection

Peripheral whole blood collection, two tubes of 10 mL per subject, was performed via venipuncture and leukocytes were separated through a washing protocol. The blood was mixed well and centrifuged at 2800 rpm for 10 min, 4° C. The white cell phase was collected and mixed with Cell Lysis Buffer. After 10 min at room temperature, the samples were centrifuged at 1800 rpm for 10 min, 4 °C. Distilled water was mixed to the obtained leukocytes' pellet and centrifuged at 1800 rpm for 10 min, 4 °C. Saline solution (0.9%) was finally added to the leukocytes' pellet. A DNA purification protocol for leukocytes was performed using QIAamp DNA Blood Midi/Maxi Kit (Spin Protocol, QIAGEN) at the Galician Public Foundation of Genomic Medicine of the University of Santiago de Compostela (Spain). Aliquots of the genomic DNA extracted were sent to Aalborg University, Denmark, for the present epigenetic study.

DNA methylation analysis

A targeted DNA methylation analysis was conducted through a customized SureSelect^{XT} Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library (SureSelect^{XT} cat # 5190-4806 and #G651A, Agilent Technologies, Santa Clara, CA), a single-base resolution approach, based on bisulfite conversion [46]. The protocol included five main steps. i) *Capture library preparation:* a pool of synthetic RNA fragments complementary to the selected genome regions, 4818 probes in total, were synthesized for a genome coverage of 240.134 kbp using the Agilent SureDesign tool. The selected regions of interest were related to CpGs islands, promoters and transcription start sites of 100 genes (Table S3), identified using the UCSC genome browser (on Human Dec. 2009 (GRCh37/hg19) Assembly; https://genome-euro.ucsc.edu/cgi-bin/hgGateway). The 'Nucleotide' molecular database (National Library of Medicine, US) and the Ensembl genome browser (www.ensembl.org, n.d.) were used to determine the exact DNA sequences to be included in the library and to explore genes information. ii) *gDNA shearing:* samples quantified using Qubit and DNA (3 µg for each subject) were sonicated with Covaris® S2 system (Covaris, Woburn, MA) to obtain products of 150–250 bp. iii) *Hybridization and capture*: DNA was first end-repaired, A-tailed and ligated with paired-end methylated adapters to create a pre-capture DNA library, using SureSelect^{XT} Library Prep Kit ILM; sample purifications were conducted using AMPure XP beads and magnetic separator devices. The DNA samples (500 ng) were then hybridized to the RNA SureSelect Human methyl-seq capture library at 65 °C for 16 h and hybridized products were captured using streptavidin-coated beads. iv) *Bisulfite conversion* of the purified hybridized products (64 °C for 2.5 h) using the Zymo EZ DNA Gold kit (Cat #D5005, Zymo Research, Irvine, CA) and the bisulfite-treated library was PCR-amplified for 14 cycles. v) *Sequencing*: the captured libraries were finally amplified with 8 bp indexing primers appropriate for each sample (six cycles PCR); the indexed samples (4 ng/µL) were clustered at 12 pM denatured libraries on a V3 paired-end read flow cell (MiSeq Reagent Kits v3) and sequenced for 600 cycles on an Illumina MiSeq platform.

Bioinformatic data processing: bisulfite-treated 200 nt-long paired-end sequencing reads were aligned to the human reference genome (GRCh37/hg19) using BS-Seeker2 [47] with standard parameters. Each mate of the pair was mapped in single-end mode and the resulting alignments merged, as recommended. Conversion of the alignment files to the CGmap format has been performed using CGmapTools [48] with the -rmOverlap option to remove overlapping regions of two mate reads avoiding dual consideration of the same DNA fragment. Finally, Metilene [49] was used for the identification of differentially methylated regions and cytosines.

Differential methylation was performed for all the cytosine environments; analysis of methylated cytosines in GCH and CHH contexts was also considered.

Statistical analyses

Metilene software, with a binary segmentation algorithm combined with a two-dimensional statistical test [49], was used for the detection of differentially methylated regions (DMRs, test parameters: -M 500 -f 2 -m 5 -d 0.01) and differentially methylated cytosines (DMCs, test parameters: -f 3-m 5 -d 0.1). In particular, the software assesses the statistical significance of potential DMRs by a two-dimensional version of the Kolmogorov–Smirnov test (KS-test) [50] and an independent Mann–Whitney U test (MWU-test). The software Metilene assesses each CpG for differential methylation (DMCs test) by using the Mann–Whitney-U test. The corresponding p-Values are reported in the output.

A logistic regression model was used to test the concurrent effect of different methylation levels on the risk of developing FM. Logistic regression is the most appropriate model to estimate the risk of a disease, given the possibility to use a dichotomous variable as a dependent variable. In this case, 0 corresponded to FM absence, one corresponded to FM presence. The set of explanatory variables (age, which was included to control for potential confounding effects, level of methylation in FM, depression, sleep and inflammation regions, respectively) checked for the influence of methylation levels on the risk of FM in some selected groups of genome regions already known as associated with either FM, depression, sleep, or inflammation. Robust standard errors were applied to the regression models in order to reduce the possible bias introduced in the estimations by heteroscedasticity. The logistic analysis was conducted in Stata/IC 15.1 (StataCorp, TX 77845, USA).

It should be noted that the methylation values have been multiplied by 1000 in the model. This operation was necessary to better highlight the effects of the methylation levels on the risk of FM.

For all the statistical analyses, results were considered statistically significant for p≤0.05.

Results

DNA methylation analysis comparing cases and controls

We performed DNA methylation analysis testing for differential methylation in targeted genomic regions in leukocytes from women with FM (n= 8). Their related healthy sisters (n= 8) with similar age have been selected as controls. Siblings offer in fact a good study design to investigate the association of DNA methylation with a disease, sharing half their genome and often stable aspects of family context, allowing to reduce confounding influences due to genetic heterogeneity and potentially different prenatal exposures or early-life environmental effects.

The targeted sequences to be analyzed were related to potentially relevant genes for FM development or comorbid symptoms and were identified through a literature review. A total of 112 genomic regions belonging to 100 genes were selected, based on previous associations with FM or CWP, depression and other psychiatric disorders, inflammation, peripheral fiber innervation and sleep disorders. Regions related to chromatin regulation and miRNAs genes were also included (Tables S3, S4).

The DNA methylation analysis was performed at two levels: a differentially methylated regions (DMRs) test determining methylation by grouping neighboring cytosines and a differentially methylated cytosines (DMCs) test revealing methylation at single cytosine level.

DMRs test revealed two regions (*GRM2* and *DRD3*) with a small but significant difference in the level of DNA methylation. The results and the chromosomes' coordinates related to the two identified DMRs are listed in Table 1. The first region (chr3: 51740486–51741687) is related to the *GRM2* gene promoter and includes a CpGs island; the level of methylation resulted significantly higher (p (MWU)=1.80E-06) in FM patients (mean methylation: 0.1) than controls (mean methylation: 0.087). The *GRM2* gene encodes the Glutamate Metabotropic Receptor 2 (mGlu2) [51], that regulates the glutamatergic neurotransmission and can be perturbed in many neuropathologic conditions [52]. However, after correction for multiple comparisons the difference in methylation level remained significant using only the MWU statistical test (adjusted pval <0.0002). The second region (chr3: 113897675–113898814) is related to the *DRD3* gene promoter region encoding the dopamine receptor D3. Even in this case the level of methylation was significantly higher (p(MWU)=0.028) in the eight FM women (mean methylation: 0.030) compared with their healthy sisters (mean methylation: 0.006). However, the significance was not revealed by the KS-test and disappeared after correction for multiple comparisons with the MWU test.

Bisulfite conversion associated to NGS sequencing allowed to reveal the methylation level also at single-base resolution. Since DNA methylation can also occur in non-CpG methylation contexts in human genome, we extracted methylation levels even in CHH and CHG contexts (H=A, C or T). The analysis revealed that in CHG and CHH contexts only 1.377 and 1.289% of cytosines were methylated respectively, indicating that the methylation trend did not seem to be affected or biased by methylation levels in these contexts.

DMCs test revealed instead 23 differentially methylated cytosines (methylation difference≥10%), belonging to 10 genes, that reached statistical significance (p<0.05) (Table 2). Increased methylation in FM women compared with their healthy sisters was found in cytosines related to *SYT2*, Synaptotagmin 2; *GCSAML*, Germinal Center Associated Signaling and Motility Like; *GRM2*, Glutamate Metabotropic Receptor 2; *MAOB*, the Monoamine Oxidase B; and the oncogene *MCF2*. Decreased methylation in FM women compared with their healthy sisters was evidenced in cytosines related to *NR3C1*, Glucocorticoid Receptor; *TRPA1*, the Transient Receptor Potential Cation Channel Subfamily A Member 1; *ZNF438*, Zinc Finger Protein 438; *IL25*, Interleukin 25; and *SAMD4A*, Sterile Alpha Motif Domain Containing 4A. Ten of the 23 CpGs differentially methylated

Table 1: Metilene DMRs test output: regions in which a significant difference of at least 1% in methylation levels was found using either the MWU or the 2D KS test.

Gene	Chr	Start	Stop	#CpGs	p (MWU)	p (2D KS)	Mean methylation level CTRLs	Mean methylation level FM
GRM2	chr3	51740486	51741687	321	1.80E-06	0.074	0.087	0.100
DRD3	chr3	113897675	113898814	1	0.028	1	0.006	0.030

CTRLs, control subjects; FM, fibromyalgia patients.

Table 2: DMCs test output: cytosines in which a significant difference in methylation levels of at least 10% has been found using the MWU test.

Gene	Chr	Start	Stop	p-Value (MWU)	Mean methylation CTRLs	Mean methylation FM
SYT2	chr1	202678998	202678999	0.028	0.000	0.101
GCSMAL	chr1	247681710	247681711	0.028	0.518	0.654
	chr1	247681760	247681761	0.005	0.376	0.519
	chr1	247681781	247681782	0.015	0.396	0.516
GRM2	chr3	51740891	51740892	0.028	0.098	0.200
	chr3	51741080	51741081	0.015	0.309	0.483
	chr3	51741245	51741246	0.021	0.225	0.338
	chr3	51741346	51741347	0.038	0.319	0.445
	chr3	51741351	51741352	0.010	0.363	0.481
	chr3	51741371	51741372	0.001	0.198	0.329
	chr3	51741375	51741376	0.028	0.233	0.339
	chr3	51741412	51741413	0.038	0.264	0.391
	chr3	51741444	51741445	0.005	0.573	0.700
	chr3	51741473	51741474	0.015	0.640	0.763
NR3C1	chr5	142782750	142782751	0.001	0.149	0.009
TRPA1	chr8	72987438	72987439	0.021	0.266	0.163
ZNF438	chr10	31320877	31320878	0.038	0.179	0.076
IL25	chr14	23841510	23841511	0.010	0.148	0.016
SAMDA4	chr14	55025046	55025047	0.028	0.759	0.865
	chr14	55045290	55045291	0.001	0.775	0.898
MAOB	chrX	43741675	43741676	0.028	0.160	0.270
MCF2	chrX	138774409	138774410	0.038	0.146	0.305
	chrX	138774794	138774795	0.028	0.360	0.489

CTRLs, control subjects; FM, fibromyalgia patients.

were related to *GRM2* gene. Once again this indicates that this region is an important target among the sequences analyzed. Three DMCs were evidenced in the *GCSAML* (germinal center associated signaling and motility like) gene on chromosome 1, encoding a putative signaling protein associated with the sites of proliferation and differentiation of mature B lymphocytes [53]; two DMCs were from the *SAMDA4* gene and *MCF2* gene. The other six genes presented a single differential methylated CpG. However, when applying the correction for multiple comparisons no differences persisted in the DMCs test.

The effects of DNA methylation on FM risk

With FM being a multifactorial condition, the co-occurrence of DNA methylation variation in different genome regions may increase the risk of developing this condition more than changes in a single region. For this purpose, a logistic regression model evaluated the methylation levels in the genes grouped based on their association with FM, depression, sleep, inflammation, chronic pain, psychiatric disorders, innervation, chromatin regulation and miRNAs in relation to the risk of developing FM (Table 3A, B). A first model (not shown here) brought us to reduce the number of regions investigated after application of the Variance Inflationary Factor, which allowed highlighting the contribution of the single variables on the overall multicollinearity. The strong correlation among some of the variables led to the exclusion of the methylation levels in the regions related to chronic pain, psychiatric traits, innervation, chromatin regulation and miRNAs. The results (Table 3B) revealed that the level of methylation in the regions related to depression and inflammation were significantly associated with FM: a unit increase in the methylation level of depression-related genes corresponded to a significantly 32.57 times higher risk of suffering from FM (OR 32.57; <0.0001) compared with the related healthy sisters. On the other hand, a one-unit increase in the level of methylation in inflammation-related genes significantly reduced the risk of FM by 57% (OR 0.428; p=0.001).

Discussion

The present study selected a large number of candidate genes, including not only genes previously associated with FM but also with FM symptoms. Comparing FM with control samples, we observed differences in the methylation levels of the *GRM2* gene, which plays an important role in encoding the type-2 metabotropic glutamate receptors

Table 3: Simultaneous influence of the level of DNA methylation of genes grouped by classes on the FM risk. A) Classes by which the genes have been grouped based on previous bibliographic associations. B) Logistic regression model – explanatory variables: age, levels of methylation in the regions related to depression, FM, sleep, inflammation; dependent variable: FM (bold values denote statistical significance).

(A)

Fibromyalgia		Depression	Inflammation	Sleep	Chromatin regulation	Innervation	MiRNAs	Psychiatric traits		Chronic pain
AKAP12	MKRN1	ASTN1	CRP	CYS1	DNMT1	S1PR2	MIR106B	ADRB2	NR3C1	ADIPOR2
AKAP6	MYT1L	B3GLCT	GRM2	DLG4	HDAC1	SAMD4A	MIR129-2	ANKK1	OXT	CAMKIIA
ANK3	NRXN1	MAD1L1	HSPB6	FMR1	MECP2	–	MIR130A	ASTN1	OXTR	CLEC3B
C11ORF40	NRXN3	NBAS	IL10	KLF15	SCMH1	–	MIR145	B3GLCT	PANX1	COL1A2
C11ORF83	OPRM1	NCAM1	IL17A	PCDH19	–	–	–	CNR1	SERPINA6	EHMT2
C1ORF150	PLCE1	SGIP1	IL25	RAB11B	–	–	–	COMT	SGIP1	ESR1
CRH	RGS17	SORCS3	IL36A	WNT5A	–	–	–	DRD2	SLC6A3	GRM1
ENPP3	RGS4	TMEM106B	MAP3K8	–	–	–	–	DRD3	SORCS3	HSPB6
GABRB3	RNF11	TPH2	–	–	–	–	–	GRIN2A	TACR1	IL17A
GATA2	SHISA6	WDR26	–	–	–	–	–	GRM2	TMEM106B	KCNS1
GBP1	SLC6A4	–	–	–	–	–	–	MAD1L1	TPH2	MAOB
GCH1	STEAP2-AS1	–	–	–	–	–	–	NBAS	WDR26	MDH2
GRIA4	STIM1	–	–	–	–	–	–	NCAM1	–	REST
LMO7	TAAR1	–	–	–	–	–	–	–	–	SYT2
LOC105376481	TSPO	–	–	–	–	–	–	–	–	TNFRSF13B
MACROD2	UNC5C	–	–	–	–	–	–	–	–	TRPA1
MARCH8	ZBBX	–	–	–	–	–	–	–	–	TRPV1
MCF2	ZNF77	–	–	–	–	–	–	–	–	–

(B)

Dependent variable: FM	Odds ratio	Robust Std.Err.		p-Value>|z|
Variables in the equation				
Age	0.814	0.098		0.087
Level of methylation in *depression* genes	32.570	29.757		**0.000**
Level of methylation in *fibromyalgia* genes	1.247	0.821		0.737
Level of methylation in *sleep* genes	0.988	0.111		0.914
Level of methylation in *inflammation* genes	0.428	0.112		**0.001**
_Cons	1.20e−15	2.14e−14		0.054
Log pseudolikelihood=−3.7269713			Wald $chi^2(5)=19.57$	
			Prob>$chi^2=0.0015$	
			Pseudo $R^2=0.6639$	

(mGluR2) and a simultaneous significant association of levels of methylation of depression and inflammation-related genes with FM.

DNA from simple blood extraction might be used to develop noninvasive molecular tests. In fact, some evidence shows a blood-brain methylation correspondence [54] with specific overlapping signatures of chronic pain in DNA methylation of prefrontal cortex and peripheral T cells [55]. Targeted bisulfite sequencing analysis has been confirmed as a valid approach to overcome potential biases and to increase the accuracy of methylation quantitative measures and led us to detect both regions (DMRs) and single cytosines (DMCs) that might represent potential biomarkers for FM.

Both DMCs and DMRs tests bring the focus on the *GRM2* gene. In particular, an increased methylation level in the *GRM2* promoter region was observed in FM women compared with their healthy sisters. *GRM2* encodes the type-2 metabotropic glutamate receptors (mGluR2), an inhibitory auto-receptor that modulates glutamatergic signaling throughout the central and peripheral nervous system [56, 57] and indirectly modulates other neurotransmitters including dopamine and GABA [58]. The activation of mGlu2 and mGlu3 receptors in peripheral sensory neurons was demonstrated to be sufficient for analgesia [59, 60], and consistently, pharmacological inhibition can prolong pain-like behavior [61, 62]. The ability of mGluR2s to reverse the sensitization of capsaicin receptors and the thermal hyperalgesia induced by prostaglandin E2 suggested this receptor as a therapeutic intervention in inflammatory pain states [63, 64].

Based on this evidence, we could hypothesize (Figure 1) that the increased DNA methylation observed in FM women may in some way reflect a lower mGlu2 mRNA expression, a lower availability of mGlu2 receptors, a lower inhibition on glutamate release and an increased level of glutamate, triggering the central sensitization state found in FM patients [65–69]. Further, the potential role of *GRM2* in the FM pathogenesis is supported by its involvement in the sleep regulation pathway, proved in Grm2/3 double knockout (Grm2/3-/-) mice [70].

The DMRs test revealed another hypermethylated region in FM women compared with controls related to the *DRD3* gene. *DRD3*, encoding dopamine receptor D3, has been hypothesized to be involved in the pathophysiology of several psychiatric disorders, including schizophrenia [71]. Among the dopamine receptors, D3 receptors have the highest density in the limbic areas of the brain, which are associated with cognitive and emotional functions [72]. Disrupted dopaminergic neurotransmission is one of the hypothesized triggering mechanisms in FM development, with lower concentrations of dopamine found in FM patients in comparison with matched controls [73]. In addition, a positron emission tomography experiment showed that abnormal dopamine function may be associated with differential processing of pain perception [74]. Interestingly, studies have demonstrated direct interactions of D3 receptors at glutamatergic synapses and modulation of glutamate activity by D3 receptor blockade [71].

The DMCs analysis in FM women and controls revealed differently methylated cytosines also in other genes. In

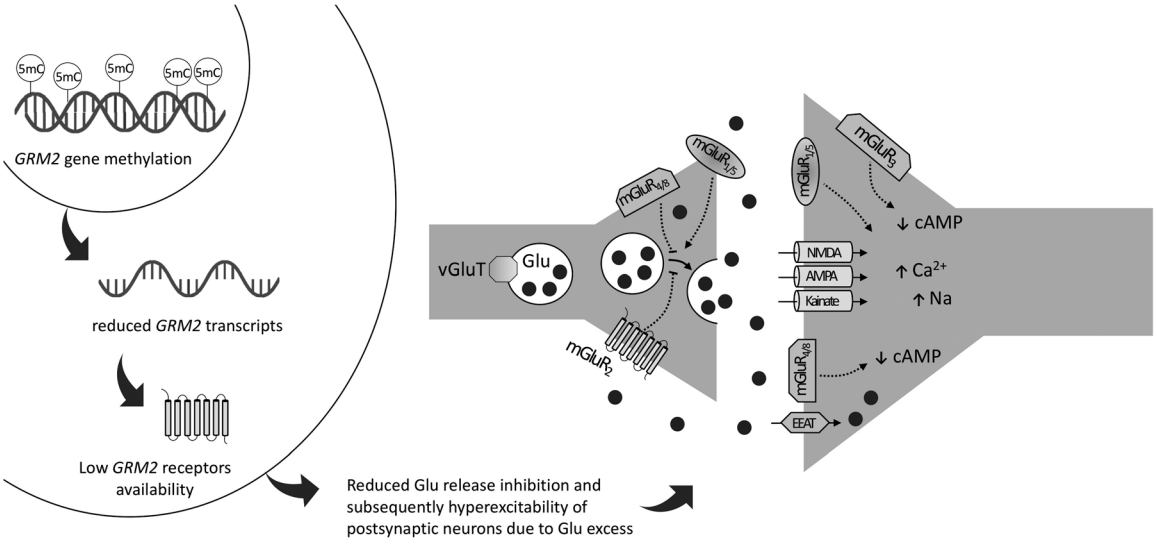

Figure 1: Hypothesis on *GRM2* hypermethylation consequences in FM patients. Glutamatergic neurotransmission key players are reported (Glu: Glutamate; mGluR: Metabotropic glutamate receptor; vGluT: Vesicular glutamate transporter; EAAT: Excitatory amino acid transporter; NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), and kainate (kainic acid) receptors).

particular, three cytosines were found differentially methylated in the *GCSAML* gene. Consistently with our results, the *GCSAML* expression was found downregulated in a genome-wide expression profiling in the peripheral blood of patients with FM [75]. Interestingly, a recent study discovered the occurrence of maternally inherited 5mCpG imprints at the DMR linked to this protein-coding gene and demonstrated its potential influence on transcription factors expression from the paternal allele [76].

Moreover, increased methylation was observed in other cytosines related to *SYT2*, already associated with long-term changes in DNA methylation in a chronic pain model [77]; *MAOB*, a potential biological candidate for pain because of the significant associations between the A/G polymorphism and postoperative perception of pain [78]; *MCF2*, the SNP *rs12556003* of which was associated with FM [79]. The genes with decreased methylation in FM women in the cytosines identified are *NR3C1*, for which a hypomethylation was found to be associated with post-traumatic stress disorder [80]; *TRPA1*, for which a hypermethylation in subjects with a low pressure pain threshold was demonstrated [81]; *IL25*, for which an upregulated expression of the inflammatory cytokines (IL10, IL25 and 1L36A) was already evidenced in FM [75].

An interesting result highlighted by the logistic regression analysis revealed the simultaneous association of methylation levels of depression and inflammation genes with FM. This strong association may support the hypothesis that a methylome pattern, a combination of several methylated/unmethylated genes, might represent a risk factor to FM or a consequence of the disease, more than single genes. The DNA methylation pattern in the regions related to depression and inflammation genes may reflect or cause the complex phenotype of FM susceptibility (Figure 2). An increased level of methylation in the analyzed sites of depression-related genes should be reflected in a lower expression of these genes that are involved in excitatory neurotransmission as in the serotoninergic pathway, mechanosensory behavior, post synapse, neuron spine and dendrite functions [82]. A decreased level of methylation in inflammation-related genes should be associated with a higher level of their expression, consistently with the upregulation of several inflammatory pathways previously evidenced in FM patients [75]. A higher level of inflammation and a higher level of depression in FM patients may lead to highlight the complex interplay between physical and psychological exhaustion in which subjects suffering from FM are held.

Concerning the role of depression and inflammation genes, our results recall the bidirectional communication between the brain and the immune system [83]. Immune cells, including lymphocytes, express neurotransmitter receptors that allow the interaction with circulating

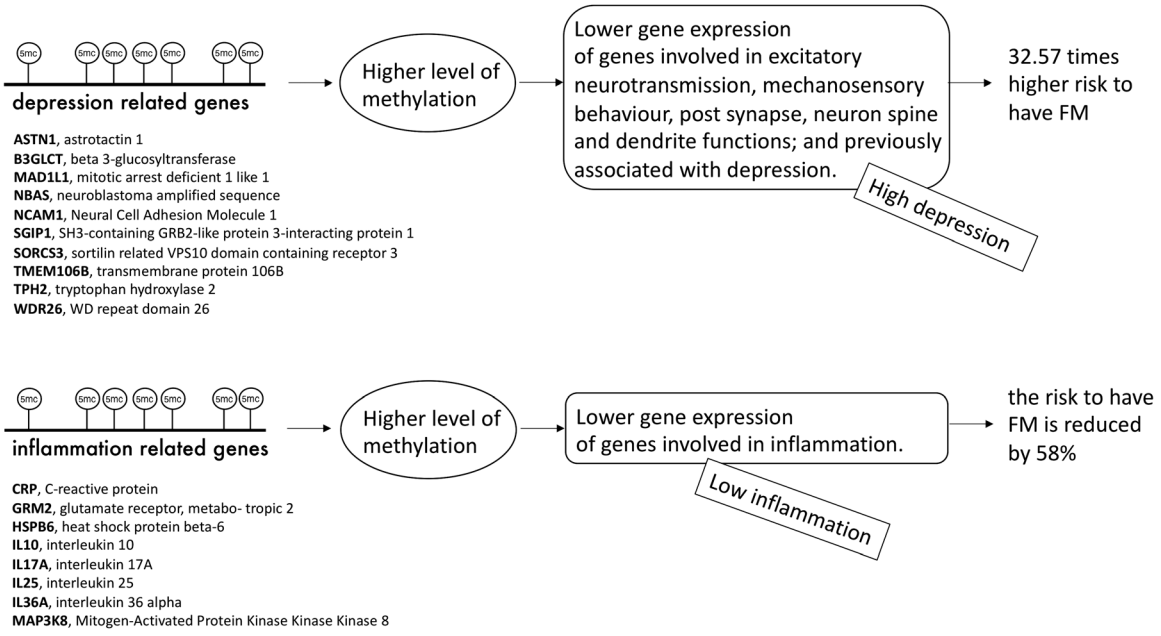

Figure 2: Hypothesis on the effect of DNA methylation changes in depression and inflammation genes. DNA methylation changes in genes previously associated to depression and inflammation were found significantly associated with FM in the logistic regression model comparing eight FM women and their eight healthy sisters.

neurotransmitters and neurochemicals. Thus, signals from the brain can influence the immune system in response to a changing external environment, including psychological and physiological stress [84]. In turn, immune cells produce signaling molecules including catecholamines and cytokines acting in the neuro-immunomodulatory circuitry [85–87]. The fact that FM may be a disorder associated with immune dysregulation is also supported by the IgG deficiency observed in FM patients [88, 89]. In addition, low-dose naltrexone, observed to modulate the immune system function of the body to resist an abnormal immune response [90], has been demonstrated to reduce symptom severity also in FM, supporting the notion of immune modulation and glia cell modulation in FM patients [91]. Inflammatory cytokines may reflect the brain and immune system interaction resembling the core symptoms of FM and other Central Sensitivity Syndromes [89]. These interactions affect also the circadian rhythms potentially explaining the sleep disorders associated with FM [92].

Although the individual differences in methylation levels are rather small, the identified signatures in the blood cells should be taken into account to verify them on a larger subset of subjects and for future epigenetically based biomarker development.

The present study has certain limitations. First, the low number of samples highlights the need to confirm the results by increasing the number of patients and controls. A second limitation of the study is that psychosocial factors have been observed to represent more than a simple association with FM but appear as factors that mainly contribute to the risk condition. The lack of information concerning early life events or stressor experiences does not permit us to evaluate the association of traumatic stress on FM development. Moreover, given that sex-related differences in DNA methylation levels have been previously reported [93, 94], we performed the present pilot study only including a sample of women. We acknowledge this may be a limitation, concerning the generalization of results to male patients with FM. Another limitation is the low starting material that did not allow a transcriptional analysis. Therefore, the relationship between DNA methylation and mRNA levels should be examined in future replication studies. In addition, most of the changes in DNA methylation patterns were observed in peripheral cells that may not reflect changes in central pain mechanisms. Replication studies using specific brain and dorsal root ganglia tissues should help to further clarify the role of DNA methylation in FM. Finally, it is not possible to establish any causal relationship on the differences highlighted. Future longitudinal designs including both environmental and methylation data might clarify the specific causal relations between the factors involved, revealing if they are linked to the etiology of the disease.

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