A family-based study to identify genetic biomarkers of fibromyalgia: consideration of patients' subgroups

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ABSTRACT

Objective. *Evidence from genome-wide and candidate gene association studies, familial aggregation and linkage analyses demonstrate the genetic contribution to fibromyalgia (FM) disease. This study aimed to identify genetic biomarkers of FM and its related comorbid disorders, by exploring 41 polymorphisms potentially involved in FM pathogenesis in families with at least one patient with FM.*

Methods. *Core symptoms were assessed, and blood samples collected from 556 patients with FM and 395 healthy relatives. For the genetic study, a final sample of 401 FM patients and 232 healthy controls was selected, discarding patients with concomitant pathologies and controls with chronic pain. A family-based approach using DFAM test (Plink) and SNPs (single nucleotide polymorphisms) combination analyses to compare FM patients vs. controls were first applied. Second, the genotypic distribution of subgroups of FM patients, stratified by severe vs. mild symptoms of pain, depression and sleep impairment, was considered.*

Results. *No evidence of associations with FM per se were detected, using either a family-based approach or SNPs combination analyses. However, considering the subgroups of FM patients, the SNP rs6454674 (CNR1, cannabinoid receptor 1 gene) was found as a potential genetic marker of FM correlated with depression ($p<.001$).*

Conclusion. *No significant associations using either the family-based analysis or the SNPs combination tests dissociated FM patients and their healthy relatives. FM patients with and without depression showed a significant difference in the genotypic distribution related to the SNP rs6454674 in the cannabinoid receptor 1 gene (CNR1) indicating that FM is not a homogenous disorder.*

Introduction

Fibromyalgia (FM) is a multifactorial disorder characterised by chronic widespread pain, fatigue and cognitive impairments. Its prevalence of 2 to 6% in the adult population (1-3), along with its high functional impact and comorbidities (*e.g.* depression, sleep disturbances) makes FM a health problem with substantial socio-economic effects (4-6). Thus, research is essential to characterise pathophysiological mechanisms underlying its development and maintenance.

The heritability component of FM has been assessed in twins and family studies, showing familial aggregation among FM patients (7, 8). Linkage studies (9) and whole exome sequencing in nuclear families (10) also highlighted the genetic predisposition in FM. Several candidate gene association studies have identified gene variants potentially affecting the pathophysiology of the disease, mostly in the dopaminergic, serotoninergic (11-13) and catecholaminergic systems (14). Some genetic variants supported a role for the immune system (15), consistently with immune system abnormalities found in FM patients (16), but positive associations are not always confirmed (17). In addition, genome-wide association studies (GWAS) revealed variants in genes that are essential players for neuronal development and previously associated to brain dysfunctions (18). The association of *GRIA4*, encoding a glutamate receptor subunit, suggests the role of central sensitisation in FM, possibly caused by hyperexcitability of glutamatergic receptors (19). Significant associations with *TAAR1*, *RGS4*, and *CNR1* have focused the attention on the endocannabinoid system, consistently with the increased circulating endocannabinoid anandamide found

in FM patients (20). Moreover, associations with polymorphisms related to interleukins and the endogenous opioid system suggest the involvement of chronic inflammation in FM (21-24).
In spite of some positive evidence of association of these systems with FM syndrome, the results are far from being consistent. Most of the genetic studies present some methodological limitations, such as the small number of individuals and incomplete characterisation of patients' status. In addition, many studies have not considered that, in multifactorial conditions like FM, SNPs combinations may be associated to the disease risk more than single variants. To overcome those troublesome issues, the present study aims to 1) characterise and stratify a large cohort of FM patients and their healthy relatives, 2) explore possible genetic markers associated with FM through a family based-approach and SNPs combination analyses, and 3) investigate possible genetic differences associated to specific FM symptoms/comorbidities (pain, depression, sleep disturbances), in order to characterise not only FM per se but subgroups of patients with their unique clinical picture. For these purposes, a pool of SNPs which was associated with FM or its symptoms in previous studies was tested and a complete clinical characterisation of the cohort was performed.

Materials and methods

Subjects

A cohort of 950 Caucasian participants, including 556 patients with FM and 395 healthy relatives from their nuclear families, was enrolled. To ensure sufficient statistical power, simulations with the Quanto software (25) were performed to identify the number of families needed to ensure sufficient statistical power and the number required was established around 500. Inclusion criteria for the patients' group were FM diagnosis provided by a primary care physician or by a professional specialist in rheumatology or neurology. Exclusion criteria for the healthy participants were FM diagnosis or presence of any other chronic pain disease. The study design was approved by the Ethics Committee of Galicia, Spain (Registration Code: 2013/582) and written informed consent was obtained from all the participants, who accepted to enter in the study as volunteers.
The 556 FM patients were 99.6% females. Some of the patients participated in the study alone (123); some others were accompanied by one or more first-degree relative/s with FM (39); and the rest of participants (395) came with relatives without FM. The latter constitutes the healthy controls (HCs) group (n=395) and was composed by 89.6% females, mostly siblings.

Demographic and clinical assessment

All the participants, patients and controls, were assessed following a systematic clinical interview that included age, demographic data, medical history (including past and current diseases, family history of FM, years since diagnosis and comorbid disorders), and scales and questionnaires to assess the core FM symptoms (among them pain, depression and sleep disturbance) (See Supplementary Methods S1).
Participants were classified into six diagnostic groups, according to age and presence of pain (for HCs) and ACR 2010 criteria and comorbidities (for the patients) (Table I). For the genetic analysis, a restricted sample of HCs with low levels of pain (*i.e.* group 1, n = 232) was used and patients with FM, with and without comorbid symptoms/disorders of the syndrome (chronic fatigue, temporomandibular disorders, irritable bowel, depression), but no other pathologies (*i.e.* group 5, n=401). The 401 FM patients selected were aged 18-81 years (mean age 51±11 years) and the 232 HCs were aged 18-86 years (mean age 44±17 years).

Design

We performed a candidate gene association study of 41 SNPs including three main analyses: i) a genetic family-based study using DFAM test (a disease association analysis in families, Plink software), which allows SNPs analysis in multiple families; ii) an analysis of combination of SNPs in patients with FM and their relatives; iii) a comparison of the genotypic distribution between subgroups of FM patients (stratified by pain, depression levels and sleep dysfunction). The study follows the workflow showed in Fig. 1.

Sample collection and DNA extraction

Peripheral whole blood collection, two tubes of 10 ml per subject, was performed via venipuncture and leukocytes were separated through a washing protocol. DNA extraction from leukocytes was performed at the Galician Public Foundation of Genomic Medicine of the University of Santiago de Compostela (Spain). The washing protocol and DNA extraction are described in Supplementary Methods S2. After DNA extraction, all the samples have been quantified and divided into aliquots: aliquots concentrated 15 ng/μL were reserved for the present genetic analysis.

Genes and SNPs studied

The genetic variants included in the study were selected because their relation to FM was evidenced in previous studies or because of the relation of the corresponding functional proteins to various aspects of FM comorbidities. The forty-one SNPs selected are described in detail in Supplementary Table S1, which also reports chromosome positions, allele consequences, corresponding proteins and references of previous associations.

Genotyping

The 41 SNPs polymorphisms related to the genes listed in Supplementary Table S1 were genotyped in FM patients and controls. Genotyping was conducted by the CEGEN (Spanish National Center for Genotyping, Santiago de Compostela, Spain) using the iPlex® Gold chemistry and MassARRAY platform, according to manufacturer's instructions (Agena Bioscience, San Diego, CA; formerly Sequenom Inc.). Genotyping assays procedure is described in Supplementary Methods S2. It should be reported that the SNP rs4680 (*COMT* gene) did not survive the quality control test and therefore the results related to this polymorphism are not available.

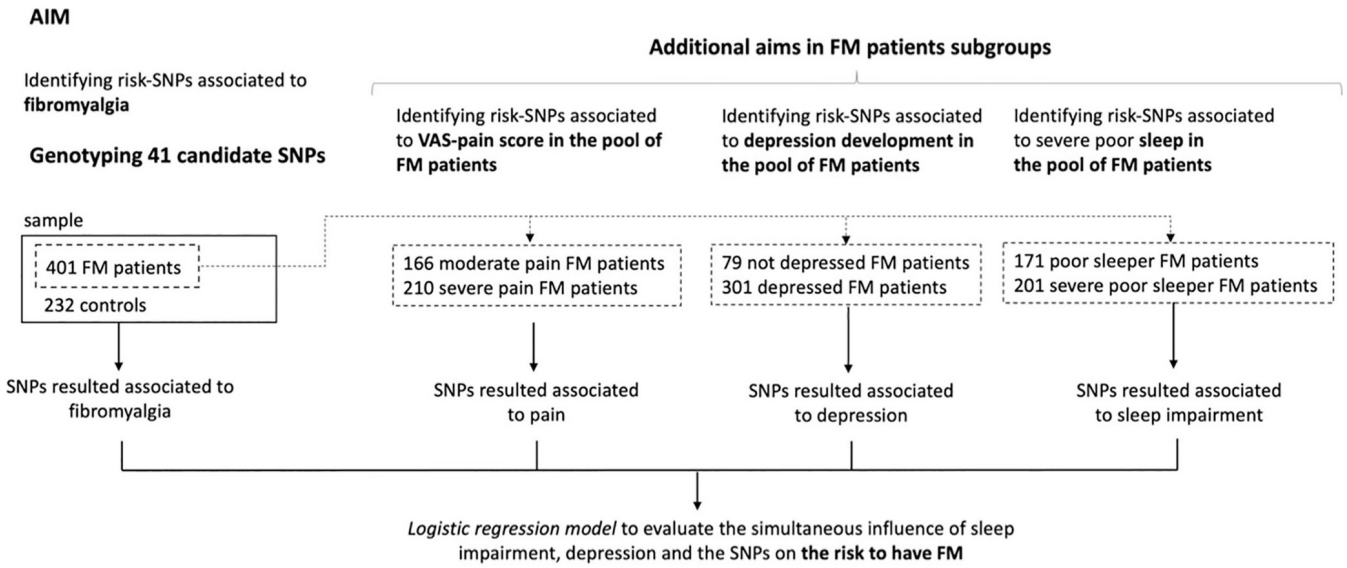

Fig. 1. Workflow diagram. The study design included a candidate gene association study in patients with FM and their healthy relatives; the same polymorphisms were also analysed considering the restricted pool of 401 FM patients (additional aims in FM patients subgroups): first comparing a subgroup of moderate pain suffering FM patients *vs*. severe pain suffering FM patients; second depressed FM patients *vs*. not depressed FM patients; and third comparing FM patients with mild sleep impairment (FM poor sleepers) *vs*. FM patients with severe sleep impairment (FM severe poor sleepers).

Statistical analyses

To generate SNP association values comparing FM patients and healthy relatives two methods were used. First, data from the 40 SNPs with minor allele frequencies >0.05 were analysed using a family-based approach using PLINK (http://zzz.bwh.harvard.edu/plink/fanal.shtml#dfam); in particular, the DFAM test was performed, an option that allows to include also sibships without parents as well as unrelated individuals. The test uses the sibling transmission disequilibrium test and by incorporating a clustered-analysis using the Cochran-Mantel-Haesnzel test, it can also include discordant sibship data, parent-offspring trio data and unrelated case/control data in a single analysis (26, 27). A threshold for nominal significance of $p<0.05$ was set.

Since genetic variants can generate a high number of combinations, co-occurring in several patients or in restricted groups, classical statistical tests may not reveal rare combinations of genetic variants significantly associated with FM. To overcome this, we analysed clusters, group of combinations sharing at least one common SNP genotype, and used permutation tests to assess whether some combinations and clusters were found exclusively in patients and thus significantly associated with FM.

Table I. Description of the diagnostic group classifications. For the present study the participants related to group 1 and group 5 (highlighted in bold) have been selected.

		Fibromyalgia patients	Healthy controls
N		556	395
Female N (%)		555 (99.6%)	353 (89.6%)
Diagnostic group classification			
1.	Control-no pain Healthy controls aged ≤45 with VAS-Pain (or FIQ-item5) <2 and healthy controls aged>45 with VAS-Pain (or FIQ-item5) between 2 and 4.		**232 (58.9%)**
2.	Control-mild pain Healthy controls aged ≤45 with VAS-Pain (or FIQ-item5)>2 and healthy controls aged>45 with VAS-Pain (or FIQ-item5) > 4.		93 (23.6%)
3.	Doubtful cases Healthy controls with chronic pain or fibromyalgia symptoms or Patients with FM diagnosis not fulfilling the ACR 2010 criteria.	4 (0.7%)	66 (16.5%)
4.	FM and other diseases Patients with diagnosis of FM and other diseases (*i.e*. self-immune, cancer, multiple scleroses) that may explain pain or fatigue symptoms.	130 (23.3%)	
5.	FM and comorbidities Patients with diagnosis of FM, with and without comorbid symptoms/disorders (chronic fatigue, temporo-mandibular disorders, irritable bowel, depression).	**401 (72.2%)**	
6.	Unclassified participants due to incomplete clinical data.	21 (3.8%)	4 (1.0%)

Finally, we stratified the patients according to pain, depression and sleep disorders, and analysed possible differences in their genotype distributions comparing the subgroups. Fisher's exact tests were applied to determine whether the genotypic distributions differ significantly between i) FM patients based on their VAS-Pain score as moderate or severe, ii) FM patients with depression (BDI score ≥14) and FM patients with no depression (BDI score <14), iii) FM patients with mild sleep impairment (PSQI score <15) and FM patients with severe sleep impairment (PSQI score ≥15) (28). PSQI

cutoff was set up according to the percentile's values calculation.

In addition, a logistic regression model was performed. The model tested the roles of the various SNPs' genotypes (only those which showed any association to FM or its comorbidities), depression and sleep quality on the risk to be suffering from FM. In this model, the dependent variable assumes the value 0 or 1 according to whether the patient does not have or have FM. Robust standard errors were applied to 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).

For all the statistical analyses, results were considered statistically significant for $p \leq 0.05$.

Results

Primary case/control genetic associations

The family-based approach, used to explore associations of the selected SNPs comparing FM patients with healthy relatives, evidenced no significant associations as displayed in the Plink output (Table II). The observed genotypes in the subjects did not differ significantly from those expected from the Hardy-Weinberg equilibrium ($p>0.05$). SNPs combination tests were used to test the large number of possible genetic variant combinations common to many patients. Shared SNP genotypes were grouped into clusters and tested statistically: single combinations of SNP genotypes significantly associated to FM were not found; however, a combination of two SNPs genotypes, heterozygous CG genotype related to *rs4453447* (*GABRB3*) and homozygous TT genotype related to *rs7147705* (*NRXN3*), was found in 17 FM patients and zero controls with *p*-value = 0.065 (Table III).

Genotypic distributions in subgroups of FM patients

The candidate SNPs were then examined in subgroups within the pool of 401 FM patients. Using VAS scores of pain, moderate (3.5 to 7.4, n=166) *vs.* severe pain suffering (7.5 to 10, n=210) FM patients were compared. The number of mild pain suffering FM patients was too low to be included (0.5 to 3.4, n=15). Fisher's exact test revealed no differences in the genotypic distributions among these two groups.

Then, a subgroup of FM patients with depression (BDI score ≥14; n=301) and another with no depression (BDI score <14; n=79) were identified. Significant associations with the studied SNPs are reported in Table IV. Adjusting the threshold for significance for the number of SNPs tested to $p<.00125$ (0.05/40 SNPs = 0.00125), the strongest association was evidenced for the SNP *rs6454674*, an intronic variant related to *CNR1* gene, encoding the CB-1 cannabinoid receptor ($p<.001$). Heterozygous G/T genotypes are more frequent in FM patients with depression and less distributed in FM patients

Table II. Family based association between FM patients and controls using SNPs potentially associated with FM development and susceptibility.

Chr	SNP	Minor allele code	Major allele code	Number observed minor alleles	Number of expected minor alleles	CHISQ	*p*
1	*rs7911*	G	A	344	353.1	1.183	0.2768
1	*rs10799897*	G	A	355	363.3	0.9674	0.3253
2	*rs11127292*	T	C	74	78.39	0.7438	0.3884
2	*rs2194390*	G	A	80	80.52	0.01009	0.92
2	*rs11126630*	T	C	382	387	0.3521	0.5529
3	*rs2087017*	G	A	303	312.6	1.335	0.2479
3	*rs11923054*	C	T	290	296.1	0.5496	0.4585
4	*rs265015*	A	G	72	69.74	0.22	0.6391
6	*rs9381682*	A	G	91	88.12	0.289	0.5908
6	*rs6454674*	G	T	219	218.7	0.00131	0.9711
6	*rs8192619*	A	G	33	31.7	0.1519	0.6967
6	*rs10782344*	T	G	169	166.1	0.1753	0.6754
7	*rs12704506*	G	A	179	176.9	0.08964	0.7646
10	*rs12770855*	T	C	71	67.84	0.4413	0.5065
10	*rs793108*	T	C	344	340.5	0.1799	0.6714
10	*rs10821659*	A	G	290	287.8	0.07099	0.7899
10	*rs1998709*	A	C	327	318.3	1.11	0.292
10	*rs2901761*	A	G	290	284.7	0.4319	0.5111
11	*rs11602757*	G	A	75	74.81	0.001451	0.9696
11	*rs79448530*	T	C	47	50.72	0.7965	0.3721
11	*rs642544*	G	T	278	282.8	0.3439	0.5576
12	*rs2701106*	C	T	298	300.5	0.09339	0.7599
12	*rs7963168*	T	C	381	382.3	0.02357	0.878
13	*rs9565180*	T	C	171	174.3	0.2205	0.6387
14	*rs981524*	C	T	176	180.5	0.4015	0.5263
14	*rs12146962*	T	C	353	350	0.1312	0.7172
14	*rs4901530*	G	A	331	321.4	1.327	0.2493
14	*rs809*	T	C	377	371.5	0.4239	0.515
14	*rs10129666*	G	A	330	340.5	1.56	0.2116
14	*rs7147705*	T	C	276	275.8	0.000714	0.9787
15	*rs4453447*	C	G	99	97.63	0.05968	0.807
15	*rs4906902*	G	A	113	118.6	0.8388	0.3597
17	*rs12601358*	G	T	105	107.1	0.1359	0.7124
17	*rs17512210*	G	T	270	267.5	0.09405	0.7591
19	*rs35699176*	A	G	25	25.99	0.1072	0.7433
20	*rs6043433*	A	G	107	115.4	1.954	0.1621
20	*rs6131711*	A	C	326	318.9	0.7335	0.3917
22	*rs6971*	A	G	228	220	1.11	0.292

Table III. The combinations of two SNP genotypes found in 17 cases and 0 controls (p=0.065).

SNP id - gene	SNP function	Chr	Chr position	MAF	PubMed ID
rs4453447 - GABRB3	G/C intron variant	15	26758325	0.184904	21905019
rs7147705 - NRXN3	T/C intron variant	14	79535753	0.49381	22959728

with no depression. This association survived the correction for multiple comparisons.

Evidence for association were also observed for other SNPs: i) for *rs11127292* of *MYT1L* (p=0.004), Fisher's Exact test revealed a higher frequency of heterozygous C/T carriers among FM patients with no depression compared with depressed FM subjects; ii) concerning *rs12770855* (*ZNF438*, p=0.04), the frequency of homozygous C/C was lower in FM patients with no depression compared with depressed FM patients; iii) a higher frequency of G/G carriers were observed in FM depressed patients for the SNP *rs6043433* (*MACROD2*, p=0.013); iv) finally, for *rs8192619* a higher distribution of heterozygous G/A subjects was found in depressed FM subjects compared with FM subjects with no depression (*TAAR1*, p=0.016). In spite of those positive results, none of them survived the correction for multiple comparisons.

In addition, 2 subgroups of FM patients were identified with different levels of sleep quality, based on their PSQI percentile values: scores <15 (n=171) as mild sleep impairment and scores ≥ to 15 (n=201) as severe sleep impairment. Fisher's exact test revealed significant differences between these subgroups in the genotypic distribution of the SNP *rs8192619* (*TAAR1*, p=0.003), with a higher distribution of heterozygous G/A subjects in FM subjects with severe sleep impairment. Concerning *rs793108* of *ZNF438*, a higher frequency of heterozygous C/T genotypes was found among mild sleep impairment patients (p=0.046). However, the result did not survive the correction for multiple comparisons.

Gene-FM comorbidities interactions

Finally, a logistic regression model (Table V) of the risk of developing FM was carried out in relation to depression (BDI), sleep disorders (PSQI) and all the SNPs previously found significantly associated with FM or FM comorbidities (*rs4453447-GABRB3, rs7147705-NRXN3, rs11127292-MYT1L, rs12770855-ZNF438, rs6043433-MACROD2, rs6454674-CNR1, rs8192619-TAAR1, rs793108-ZNF438*). Depression and sleep disorders were significantly associated with FM: a unit increase in BDI scale corresponds to 1.2% higher risk to have FM; a unit increase in PSQI scale corresponds to 1.3% higher risk to have FM. Concerning the SNPs included in the model, only the SNP *rs8192619* (*TAAR1*) resulted significantly associated with the risk to develop FM: individuals with the homozygous genotype A/A are 8.47 times more likely to have FM compared to those with the G/G genotype.

Table IV. Genotypic distributions are reported for the significant associations found considering the restricted pool of FM patients, inside which all the SNPs were analysed comparing, first, FM patients developing depression and FM patients not developing it; second, comparing FM patients with mild sleep impairment and FM patients with severe sleep impairment.

SNP id - gene	Genotypes	FM patients with depression % (n)	FM patients with no depression % (n)	Fisher's exact test
rs11127292 - MYT1L	TT	1.4 (4)	0 (0)	0.004
	CC	85.4 (245)	70.1 (54)	
	CT	13.2 (38)	29.9 (23)	
rs12770855 - ZNF438	TT	0 (0)	2.6 (2)	0.04
	CC	83.4 (242)	77.9 (60)	
	CT	16.6 (48)	19.5 (15)	
rs6043433 - MACROD2	GG	81.7 (237)	66.2 (51)	0.013
	AA	6.2 (18)	10.4 (8)	
	GA	12.1 (35)	23.4 (18)	
rs6454674 - CNR1	GG	6.2 (18)	14.3 (11)	0.001
	TT	50 (145)	63.6 (49)	
	GT	43.8 (127)	22.1 (17)	
rs8192619 - TAAR1	GG	90 (261)	96.1 (74)	0.016
	AA	0 (0)	1.3 (1)	
	GA	10 (29)	2.6 (2)	
SNP id - gene	Genotypes and alleles	FM patients with mild sleep impairment % (n)	FM patients with severe sleep impairment % (n)	Fisher's exact test
rs8192619 - TAAR1	GG	95.7 (157)	87.7 (171)	0.003
	AA	0.6 (1)	0 (0)	
	GA	3.7 (6)	12.3 (24)	
rs793108 - ZNF438	CC	23.8 (39)	34.4 (67)	0.046
	TT	18.3 (30)	20 (39)	
	CT	57.9 (95)	45.6 (89)	

No significant associations comparing FM patients and their healthy relatives

Our study does not provide evidence of association with FM for any of the 41 SNP genotypes studied. Although previous studies comparing cases and controls suggested significant genetic contributions to FM (18, 21), it is important to note that others failed to confirm SNPs specifically associated to FM susceptibility, showing weak to no association (13, 21, 29). Unlike these studies, our research included a family-based approach in a large cohort of participants, who underwent a complete assessment of FM core symptoms and comorbidities, and was performed in the Galician community of Spain: due

Discussion

The present candidate gene association study analysed 41 SNPs previously related to FM or to its comorbidities. No significant associations using either the family-based analysis or the SNPs combination tests were evidenced comparing FM patients and their healthy relatives. Comparisons of clinical subgroups of FM patients with and without co-occurring depression, showed a significant difference in the genotypic distributions related to the SNP *rs6454674* in the cannabinoid receptor 1 gene, *CNR1*.

Table V. Logistic regression model testing the simultaneous influence of depression, sleep impairments and all the SNPs found significantly associated to FM or FM comorbidities in our study (*rs11127292, rs12770855, rs6043433, rs6454674, rs8192619, rs793108, rs4453447, rs7147705*) on the risk to have FM.

Logistic regression				Number of obs = 549 Wald χ^2 (18) = 139.6 Prob > χ^2 = 0.0000	
Log pseudolikelihood = -141.18132				Pseudo R2 = 0.6091	
Fibromyalgia	Odds Ratio	Robust Std. Err.	P >|z|	[95% Conf. interval]	
BDI	1.202	0.036	0.000	1.134	1.274
PSQI	1.333	0.050	0.000	1.242	1.441
rs11127292 - *MYT1L* (ref. cat CC)					
CT	0.687	0.288	0.301	0.302	1.567
TT	1.612	1.006	0.474	0.474	5.482
rs12770855 - *ZNF438* (ref. cat CC)					
CT	1.130	0.411	0.736	0.554	2.306
TT	1.592	1.361	0.586	0.298	8.506
rs6043433 - *MACROD2* (ref. cat GG)					
AG	1.710	0.646	0.156	0.815	3.588
AA	1.731	0.802	0.236	0.698	4.296
rs6454674 - *CNR1* (ref. cat TT)					
GT	0.617	0.208	0.153	0.318	1.196
GG	1.799	1.103	0.338	0.541	5.984
rs8192619 - *TAAR1* (ref. cat GG)					
AG	1.142	0.575	0.792	0.426	3.064
AA	8.476	8.468	0.032	1.196	60.064
rs793108 ZNF438 (ref. cat CC)					
CT	1.368	0.482	0.374	0.686	2.730
TT	1.303	0.577	0.550	0.547	3.103
rs4453447 - *GABRB3* (ref. cat GG)					
CG	1.350	0.457	0.375	0.695	2.623
CC	0.397	0.467	0.433	0.039	3.982
rs7147705 - *NRXN3* (ref. cat CC)					
CT	0.798	0.263	0.496	0.418	1.525
TT	1.033	0.505	0.946	0.396	2.698
_cons	0.008	0.004	0.000	0.003	0.025

Note: _cons estimates baseline odds.

to the practically inexistent immigration until very recent times, this genetically homogenous population is thus the ideal place for the identification of genetic risk determinants (allowing to increase the power of the genetic analysis and to reduce confounding influences).

In complex diseases such as FM, rather than associations with single SNPs, rare combinations of genetic variants presumably interacting with environmental factors should be expected. Since classical statistical tests cannot capture those combinations, SNPs combination analysis was performed and found the co-occurrence of two SNP genotypes exclusively in 17 FM patients and not in healthy controls, although only with a tendency to significance (p=.065). The first SNP genotype was the heterozygous C/G of *rs4453447* related to the gamma-aminobutyric acid type A receptor β3 gene (*GABRB3*). Interestingly, gene-targeting inactivation of *GABRB3* in mice showed thermal hyperalgesia, tactile allodynia, and altered antinociception in response to analgesic drugs (30). The second SNP genotype was the homozygous T/T related to *rs7147705* in the neurexin 3 gene (*NRXN3*), an essential gene for neuronal development and for signal transmission, already found associated with FM (18). Despite this result, the number of carrier subjects of the evidenced combination is too low to allow speculating on the genes' potential role in the pathophysiological mechanisms of FM.

Genotypic distribution differences identified among subgroups of FM patients

No genotypic distribution differences among subgroups of FM patients stratified by VAS pain scores (as moderate and severe pain suffering) were found. Previous studies identified SNPs associated to pain and central sensitisation. Kosek *et al*. in Sweden showed polymorphisms in the serotonin transporter gene associated to conditioned pain modulation (CPM) and thermal pain (31, 32). Our result may be explained by the use of self-reported indices of pain and suggests the importance to use other indicators for pain and central sensitisation (33); in particular, temporal and spatial summation measurements and CPM (34, 35) could help to improve the understanding of genetic contribution to the risk of central sensitisation and FM. Also, gene-to-gene interactions in relation to endogenous pain modulation should be analysed (36).

Conversely, the consideration of subgroups of FM patients characterised by clinical phenotypes as depression and sleep impairment led us to identify some differences in the genotypic distributions. The strong significant associated SNP found comparing FM patients developing depression and FM patients not developing depression was *rs6454674* in the cannabinoid receptor 1 gene *CNR1,* an intronic T>G substitution. This variant has also been found associated to other multifactorial conditions, as Post-Traumatic Stress Disorders (37), drug addiction (38) and obesity (39). The possible *CNR1* role leads to focus on the endocannabinoid system known to regulate emotions, stress, memory, and cognition. This system has been investigated in depth in acute and chronic pain states, where systemic administration of cannabinoid receptor ligands produces analgesia in animal models (40). Modulation of the endocannabinoid 2-arachidonoyl

glycerol signalling via specific enzyme inhibitors was already hypothesised in chronic pain states (41). In addition, it has been reported that endocannabinoids cause GABAergic inhibition and dopaminergic increase in FM (42, 43) and thus alteration in this system might determine increased activity of GABAergic pathway and dopamine reduction.

We also observed genotypic differences comparing FM patients with mild sleep impairment and FM patients with severe sleep impairment: *rs8192619 (TAAR1)* and *rs793108 (ZNF438)* showed associations, but they did not survive the correction for multiple comparisons. It is interesting to note that *TAAR1,* the trace amine associated receptor 1 gene, implicated in several human conditions, including inflammation and response to infection, schizophrenia, depression, addiction, migraine, general chronic pain states, and FM, appears to act as a physiological regulator with a significant role in the modulation of CNS function and maintenance of central neurotransmission, in particular in the dopaminergic system (44). On the other hand, *ZNF438,* whose intronic variant was associated to rheumatoid arthritis in a genome-wide association study meta-analysis (45), encodes a zinc finger protein acting as a transcriptional repressor (46) and its involvement in pain has not yet been explored.

The simultaneous evaluation of the 8 SNPs which in the present study showed an association with FM or its comorbidities is essential to identify potential biomarkers of prediction. Depressive traits and sleep disorders may represent more than simple symptoms of the disease, but factors that directly contribute to this risk condition, reinforcing gene variants effects. Logistic regression model allowed to evidence these two comorbidities as crucial concurrent conditions to FM development, together with homozygous A/A genotype of *rs8192619* (*TAAR1*) that conferred higher risk to have FM, compared to G/G or A/G genotypes. The fact that we found *TAAR1* SNP associated to both depression and sleep impairments among FM patients and to FM risk development invites to further study its involvement in FM: its function in chronic widespread pain development is also supported by its ability to modulate dopamine bioavailability and activity of dopaminergic receptors and to be a modulator of glutamatergic transmission in the prefrontal cortex (44). In addition, representing a link between the CNS involvement and immune system dysregulation of FM, it might be a target for the development of novel TAAR-selective compounds with analgesic properties (47).

Limitations of the study and future perspectives

The limitations of this study include: i) even if the use of a candidate gene association study overcomes the huge number of potential targets to manage in a GWAS, this approach does not allow to identify unravelled targets contributing to FM comprehension; ii) no information concerning participants' experienced stressors have been collected. It has been well established that childhood trauma and exposure to substances of abuse may cause lasting changes in developing neurotransmitter and endocrine circuits that are linked to anxiety and stress responses (48). Recently, additional factors associated to both FM development and genetics, in particular immune system alterations, microbiota and intestinal-brain axis dysfunction, were identified (49, 50). Thus, the impact of life style and adverse experiences should be investigated simultaneously to genetics, quantitative sensory testing data, being concurrent factors in the severity of FM development later in life; iii) finally, our participants were predominantly female, so the results cannot be directly extrapolated to a male population.

Our results failed to identify single SNPs significantly associated to FM as a homogeneous disease but suggest the need to identify genetic risk factors of subgroups of patients defined by certain sub-phenotypes or comorbidities. Ablin and Buskila underscored that the evolution of the conceptual framework of FM needs also an evolution of the approach of genetic studies (51): if FM is currently understood as a continuum, defined by a heightened central processing of pain, the genetic perspective should not be longer trying to just identify genes responsible for FM as a particular entity, but rather to relate genetic factors to the characteristics of pain processing. Thus, future studies should try to identify genetic variants associated with central pain modulation mechanisms. This may lead to identify new potential pharmacological targets allowing as ultimate goal to relief symptoms of this chronic disorder.

Conclusion

This study identified a genetic biomarker (SNP *rs6454674*, *CNR1* gene) strongly associated to depression in FM patients. Other SNPs were identified in subgroups of FM patients characterised by depression and sleep disturbance. Replication of these genetic contributors could be essential for the understanding of FM molecular pathophysiology and possible differentiated interventions and indicate that people with FM compose a heterogeneous group of patients.

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