Identification of differential genetic profiles in severe forms of fibromyalgia and chronic fatigue syndrome/myalgic encephalomyelitis: a population-based genetic association study

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Abstract

Background: Fibromyalgia (FM) and chronic fatigue syndrome or myalgic encephalomyelitis (CFS/ME) are believed to be two separate illnesses that are diagnosed using separate but overlapping clinical criteria; to date there are no biological markers for either condition. The symptoms of both disorders can differ markedly in presentation, frequency and intensity and therefore it is necessary to distinguish between the subtypes. Since recent studies have begun to determine the genetic background of these diseases, the authors suggest the use of single nucleotide polymorphism (SNP) analysis to investigate their different genetic profiles.

Methods: A group of 403 women (186 FM and 217 CFS/ME) were recruited for the study using the American College of Rheumatology 1990 and the US Centers for Disease Control and Prevention (CDC) research definition for FM and CFS diagnosis criteria, respectively. The Fibromyalgia Impact Questionnaire and the CDC Symptom Inventory questionnaires were used to define severity subgroups. For each sample, 107 SNPs were genotyped by SNPlex™. An independent second

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association study with 282 women (126 FM and 156 CFS/ME) was used to validate the results.

Results: Fifteen SNPs were identified that were able to discriminate between FM and CFS patients with a likelihood ratio (LR+) of 11.5 (95% specificity). Analysis of further SNPs allowed differential genetic profiling between the most aggressive FM phenotype and the mild forms (LR+ 12.4) and between a severe CFS/ME phenotype and a milder one (LR+ 12.4).

Conclusions: In this study, the authors claim that FM and CFS/ME are, at least in a subgroup of patients, two separate diseases with an important genetic component and suggest that CFS/ME diagnosis should be an exclusion criterion for FM diagnosis. In addition, severe cases might be different disease subtypes with distinctive genetic profiles.

Introduction

Fibromyalgia (FM) and chronic fatigue syndrome or myalgic encephalomyelitis (CFS/ME) are complex diseases with important impacts on quality of life[1,2]. Although they exhibit some overlap in symptomatology, these diseases have different clinical presentation profiles as well as different criteria for case definition, prevalence and prognoses[3–5].

FM is a common chronic pain condition that affects at least 2% of the adult population in the US and other regions in the world where FM has been studied[6]; CFS/ME is a debilitating illness with no known cause or effective therapy that constitutes a major public health problem and affects 0.2–0.5% of the population[7].

For both diseases, patients exhibit considerable variation in the presentation, frequency and intensity of symptoms as well as different therapeutic responses. For these reasons, there is increased interest in validation of markers that allow the stratification and definition of subtypes[8–11]. Currently, the severity of these diseases is principally determined using autoreferenced validated questionnaires, such as the Fibromyalgia Impact Questionnaire (FIQ) for FM[12,13] and the US Centers for Disease Control and Prevention (CDC) Symptom Inventory (CSI) for CFS/ME[14]. The authors' clinical view is that the most severe forms represent different subgroups with poor prognoses and poor therapeutic responses. Best management of these would require early diagnosis, more active and integrated therapeutic attention, plus effective support both socially and in the workplace[15,16].

Thanks to the Human Genome Project, the last decade has seen revolutionary advances in human genetics. Almost 90% of the variation in the genome occurs in the form of single nucleotide polymorphisms (SNPs)[17] and they are becoming the cornerstone of genetic research. Many SNPs have no direct effect on cell function, but others currently represent a powerful method for

identifying common variants that underlie genetic predisposition to disease[18].

In this work, variation in SNPs of different aetiopathogenic pathways was used to gain insights into the genetic profiles of FM and CFS/ME. In addition, a relationship between the severity of both diseases, measured with validated tests, and the variation in SNPs was identified. The association study of SNPs was conducted to test the hypothesis that FM and CFS/ME are different clinical and genetic entities (not to distinguish FM and CFS from healthy women) and that the phenotypic variation observed in both diseases is the result of different genetic profiles.

Methods

Study design

Individuals approached to participate in the study were all registered in the Spanish 'Fibromyalgia and/or Chronic Fatigue Syndrome Patients Record' (http://www.fundacionfatiga.org/registro_pacientes.htm). In general, individuals diagnosed with FM or CFS/ME by a reference unit are invited to register in this registry and therefore it was considered a good source of subjects for the study.

In the first stage (Study 1), 2,000 patients from all over Spain diagnosed with FM or CFS/ME were invited to participate. From these, 1,371 gave written consent to take part and filled in a questionnaire that included details about their diagnosis, phenotypic characteristics, inherited diseases, familiar diagnosis of FM or CFS/ME and presence of mental disorders. These patients were also asked to answer the FIQ[12] and the CSI for CFS/ME[14] and to provide a blood sample for DNA extraction. Taking into account that there is a recognised gender bias in the FIQ[13,] eventually only women were included in the study. In addition, they had to agree to be clinically diagnosed according to the 1990 American College of Rheumatology (ACR) classification for FM[19] or the CDC criteria for CFS/ME developed by Fukuda *et al*[20] at the Hospital Clinic and CIMA Clinic (Barcelona, Spain).

In more detail, the diagnostic criteria were as follows. To obtain the FM patient cohort, the Manual Tender Point Survey recommendations were strictly observed[21]. Only rheumatologists specifically trained to perform this test took part in the diagnostic side of the study. To avoid overlapping with the CFS group, CFS diagnosis was considered an exclusion criterion for FM. Therefore, subjects who would comply with the research definition for CFS[20] were not included in this group. In their most recent evidence-based recommendations for the management of FM, the European League Against Rheumatism (EULAR) also excluded from their research all studies that incorporated CFS patients[22]. To obtain the CFS patient cohort, the research definition for CFS[20] was followed. Among the checks required for inclusion in this group, the authors had to be able to detect a physical and cognitive impairment greater than 50% compared with pre-morbid activities. For the physical impact, all patients were asked to exercise on a treadmill under a standard Bruce protocol,

as recommended by the American Medical Association for objective evaluation of abnormal fatigue[23]. Metabolic equivalent values and determination of the percentage of the theoretical maximal cardiac frequency were calculated.
To evaluate the cognitive function, a validated neurocognitive test[24] was carried out. It was accepted that there may be CFS patients without physical or cognitive dysfunction, but it was felt that the presence of those symptoms would only improve the quality of the cohort selection.

In addition, those patients who exhibited a mental illness or psychological impairment prior to the onset of either disease were excluded from the study.

Therefore, at the end of the selection process the number of recruited subjects was reduced to 403 patients (186 FM patients aged 45–54 years and 217 CFS/ME patients aged 30–39 years). This first set of patients (Study 1) was used to explore the data and develop the models. A second phase of recruitment was conducted at a later stage following the same inclusion/exclusion and diagnosis criteria as before for the purposes of validating the results obtained in Study 1. In this case, a total of 282 women (126 FM patients aged 46–54 years and 156 CFS/ME patients aged 32–41 years) were included in the study.

All participants gave written informed consent.

Stratification of disease severity

Two validated questionnaires (for FM and CFS/ME, respectively) were used to assess the level of disease severity. Neither the Checklist Individual Strength nor the Multidimensional Fatigue Inventory have been validated in a Spanish population and therefore could not be used in this study. To capture the overall effect of FM symptomatology, the FIQ with the 1997 and 2002 modifications was used to categorise FM patients. The total values ranged from 0 to 100, with higher scores representing a greater impact of the disease on the person's life. For example, the average FM patient scores 50, whereas severely afflicted patients score ≥70[12,13].
For CFS/ME patients, the CSI is the recommended tool to document the occurrence, duration and severity of the CFS/ME symptom complex[25]. Its subscale, Case Definition Score, reflects the frequency and intensity of symptoms according to the diagnostic criteria. The authors followed the evaluation criteria defined in it; the values range between 0 and 128, where 128 represents the highest severity[14].

Based on the authors' experience[26] and in accordance with other research teams, a cut-off value defining two subgroups (severe vs. mild/moderate) was applied for each disease, with the forms that most affect the patient's quality of life corresponding to the upper third of the scale (FIQ > 76 and CSI > 84)[13,14,27]. Using these criteria, 36.5% (n=68) and 14.3% (n=31) of the patients were classified as severely affected by FM and CFS/ME, respectively.

Genotyping and SNP selection

For each patient, peripheral blood (10 ml) was withdrawn in collaborating

laboratories (Centros de Extracción de Laboratorio Echevarne; http://www.echevarne.com) located around Spain and placed in ethylene diamine tetraacetic acid-treated tubes. In addition to the blood samples, the laboratories also retained the consent forms and questionnaires. The blood and questionnaires were then forwarded to the DNA National Bank (http://www.bancoadn.org) where DNA extraction was carried out. DNA was extracted with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's specifications. Genotyping was carried out by SNPlex™ technology[28] in the National Genotyping Centre (CEGEN, Barcelona, Spain). A total of 107 SNPs belonging to different genes (Table 1) were genotyped for each patient. The SNP selection was based on previous published data[26,29–33], emerging pharmacological therapies[34–36] and the authors' own research expertise. In addition, the SNPs selected had a minor allele frequency in the general Caucasian population of 0.1 (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp), a homogeneous distribution along the gene and location inside the exons or near to them. Only 'TagSNPs' ($R^2 > 0.8$) were taken into account as this gave more statistical power by reducing the degrees of freedom of the tests[37].

As a general rule, in regression analysis one should have at least 15 subjects per predictor[38]. Therefore, from the 107 SNPs genotyped, only the sets of loci most significantly associated with the phenotype under analysis were included in the stepwise logistic regression to limit the overall false-positive rate. The SNPs were chosen according to the suggestions published by Hoh *et al*[39]; a flow diagram of the selection process is shown in Figure 1. First of all, χ^2 tests were performed to test the conformity of the genetic polymorphisms under analysis with Hardy–Weinberg expectations (HWEs). Tests of HWEs were carried out for all loci among all the different phenotypes described. Only SNPs that conformed to HWE in both separate groups under analysis were included in the study. SNPs with extremely high deviations from the predictions of HWE (p-values <0.01) were excluded from the analysis since such deviations could indicate problems such as genotyping errors.

In addition, single locus association tests between SNP allele frequency (allelic associations) and patient status were carried out using the standard contingency χ^2 test, and p-values were determined, including Bonferroni correction for multiple testing.

The possibility that deviations from HWE in the overall population (both phenotypes under analysis together) could be important in disease causation was also investigated by combining the effect of the allelic association and total HWE. The product of the HWE p-value and the allelic association p-value was used to rank the SNPs in order of importance. The ones with the smallest p-values were included in the regression analysis.

Table 1. SNPs genotyped, their associated genes, chromosomal location and function and allelic associations.

Gene name	*Chromosomal location*	*Gene function*	*Gene symbol*	*SNP number*	χ^2 *p-value*		
					FM vs. CFS/ME	*FM: FIQ >76*	*CFS/ME: CSI >84*
Ankyrin repeat and kinase domain	11q23.1	Kinase activity	*ANKK1*	rs1800497	0.809	0.023	0.005
Armadillo repeat gene deletes in velocardiofacial syndrome	22q11.21	Member of the catenin family	*ARVCF*	rs165815	0.000	0.000	0.055
Chr. 10 open-reading frame 18	10p15.1		*C10ORF18*	rs2797489	1.000	1.000	1.000
Calcium channel, voltage-dependent, α-1A subunit	19p13.2–p13.1	Voltage-gated calcium channel activity	*CACNA1A*	rs16016	0.000	0.869	0.000
				rs16025	0.519	0.316	0.695
				rs16022	0.797	0.280	0.495
Calcium channel, voltage-dependent, α-1B subunit	9q34	Voltage-gated calcium channel activity	*CACNA1B*	rs2124655	0.002	0.000	0.000
				rs936249	0.037	0.003	0.978
				rs2229948	0.557	0.256	0.576
Catechol-*O*-methyltransferase	22q11.21–q11.23	Catechol *O*-methyltransferase activity	*COMT*	rs4680	0.000	0.001	0.012
				rs165774	0.000	0.003	0.392
				rs4633	0.002	0.043	0.261
				rs4646312	0.021	0.076	0.582
				rs740602	0.900	0.414	0.453
				rs933271	0.000	0.471	0.637
Corticotropin-releasing hormone receptor 1	17q12–q22	Corticotrophin-releasing factor receptor activity	*CRHR1*	rs7209436	0.004	0.074	0.314
				rs242924	0.010	0.006	0.000
				rs173365	0.773	0.104	0.204

SNP, single nucleotide polymorphism; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; FIQ, Fibromyalgia Impact Questionnaire; CSI, US Centers for Disease Control and Prevention Symptom Inventory Short Form, Case Definition Score; Chr., chromosome.

Table 1. SNPs genotyped, their associated genes, chromosomal location and function and allelic associations (continued).

Gene name	*Chromosomal location*	*Gene function*	*Gene symbol*	*SNP number*	χ^2 *p-value*		
					FM vs. CFS/ME	*FM: FIQ >76*	*CFS/ME: CSI >84*
Corticotropin-releasing hormone receptor 2	7p15.1	Corticotrophin-releasing factor receptor activity	*CRHR2*	rs2284217	0.000	0.033	0.000
				rs2267710	0.076	0.850	0.067
Dopamine β-hydroxylase (dopamine β-monooxygenase)	9q34	Monooxygenase activity	*DBH*	rs129882	0.225	0.379	0.573
				rs6271	0.593	0.214	0.606
				rs5320	0.897	0.938	0.209
				rs4531	0.930	0.023	0.273
				rs1076150	0.961	0.000	0.007
Dopamine receptor D1	5q35.1	Dopamine receptor activity	*DRD1*	rs2168631	0.000	0.255	0.028
				rs703748	0.000	0.005	0.994
				rs686	0.671	0.077	0.997
				rs5326	0.955	0.214	0.678
Dopamine receptor D2	11q23	Dopamine receptor activity	*DRD2*	rs1125394	0.000	0.675	0.804
				rs6278	0.000	0.010	0.802
				rs4648317	0.008	0.005	0.066
				rs1799978	0.012	0.057	0.647
				rs4586205	0.582	0.095	0.081
				rs6277	0.076	0.007	0.001

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Table 1. SNPs genotyped, their associated genes, chromosomal location and function and allelic associations (continued).

Gene name	*Chromosomal location*	*Gene function*	*Gene symbol*	*SNP number*	χ^2 *p-value*		
					FM vs. CFS/ME	*FM: FIQ >76*	*CFS/ME: CSI >84*
Dopamine receptor D3	3q13.3	Dopamine receptor activity	*DRD3*	rs324029	0.000	0.000	0.182
				rs3773678	0.002	0.593	0.001
				rs324031	0.314	0.118	0.193
				rs6280	0.849	0.088	0.472
				rs963468	0.930	0.661	0.116
Dopamine receptor D4	11p15.5	Dopamine receptor activity	*DRD4*	rs11246226	0.000	0.000	0.001
				rs3758653	0.204	0.225	0.095
Dopamine receptor D5	4p16.1	Dopamine receptor activity	*DRD5*	rs6282	1.000	1.000	1.000
5-Hydroxytryptamine (serotonin) receptor 2A	13q14–q21	Serotonin receptor activity	*HTR2A*	rs2770296	0.000	0.000	0.000
				rs6306	0.273	0.669	0.023
				rs1923884	0.635	0.005	0.485
				rs6314	0.738	0.246	0.832
				rs1058576	1.000	1.000	1.000

SNP, single nucleotide polymorphism; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; FIQ, Fibromyalgia Impact Questionnaire; CSI, US Centers for Disease Control and Prevention Symptom Inventory Short Form, Case Definition Score; Chr., chromosome.

Table 1. SNPs genotyped, their associated genes, chromosomal location and function and allelic associations (continued).

Gene name	*Chromosomal location*	*Gene function*	*Gene symbol*	*SNP number*	χ^2 *p-value*		
					FM vs. CFS/ME	*FM: FIQ >76*	*CFS/ME: CSI >84*
5-Hydroxytryptamine (serotonin) receptor 2C	Xq24	Serotonin receptor activity	*HTR2C*	rs4911871	0.000	0.000	0.035
				rs489736	0.000	0.280	0.000
				rs3813928	0.005	0.190	0.077
				rs2428721	0.009	0.007	0.584
				rs518147	0.123	0.487	0.003
				rs539023	0.196	0.334	0.677
				rs10875535	0.268	0.004	0.093
				rs475717	0.508	0.246	0.037
				rs6318	0.524	0.925	0.602
				rs12560109	0.909	0.213	0.243
5-Hydroxytryptamine (serotonin) receptor 2B	2q36.3–q37.1	Serotonin receptor activity; protein binding	*HTR2B*	rs10194776	0.000	0.000	0.047
				rs4973377	0.002	0.455	0.088
				rs1549339	0.084	0.421	0.796
				rs6437000	0.905	0.003	0.930
Interleukin-10	1q31–q32	Cytokine activity; interleukin-10 receptor binding	*IL10*	rs3024498	0.000	0.033	0.123
				rs3024510	0.000	0.730	0.558
				rs1800871	0.009	0.149	0.330
				rs3024496	0.086	0.041	0.017
				rs3024494	0.196	1.000	0.000
				rs1518111	0.475	0.001	0.106

SNP, single nucleotide polymorphism; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; FIQ, Fibromyalgia Impact Questionnaire; CSI, US Centers for Disease Control and Prevention Symptom Inventory Short Form, Case Definition Score; Chr., chromosome.

Table 1. SNPs genotyped, their associated genes, chromosomal location and function and allelic associations (continued).

Gene name	*Chromosomal location*	*Gene function*	*Gene symbol*	*SNP number*	*χ^2 p-value*		
					FM vs. CFS/ME	*FM: FIQ >76*	*CFS/ME: CSI >84*
Interleukin-6 (interferon β_2)	7p21	Cytokine activity; interleukin-6 receptor binding	*IL6*	rs1800797	0.000	0.879	0.219
				rs2069845	0.000	0.804	0.907
				rs1800795	0.027	0.415	0.555
				rs2069840	0.049	0.871	0.034
				rs2069827	0.086	0.746	0.003
				rs1474347	0.446	0.219	0.000
				rs1800796	0.983	0.208	0.434
Galectin-9 short isoform	17q11.1	Galactose binding	*LGALS9*	rs4795856	0.078	0.155	0.058
	10		*LOC387649*	rs11008581	0.067	1.000	0.000
Lymphotoxin alpha	6p21.3	Cytokine activity	*LTA*	rs2229094	0.000	0.682	0.335
Monoamine oxidase A	Xp11.3	Amine oxidase activity	*MAOA*	rs979606	0.000	0.020	0.169
				rs979605	0.002	0.456	0.674
Nitric oxide synthase 2A	17q11.2–q12	Nitric oxide synthase activity	*NOS2A*	rs2297518	0.001	0.149	0.288
				rs1137933	0.029	0.159	0.042
				rs8072199	0.155	0.000	0.341
Nuclear receptor subfamily 3, group C, member 1	5q31.3	Glucocorticoid receptor activity	*NR3C1*	rs6188	0.000	0.102	0.000
				rs6196	0.000	0.008	0.060
				rs852977	0.018	0.681	0.115

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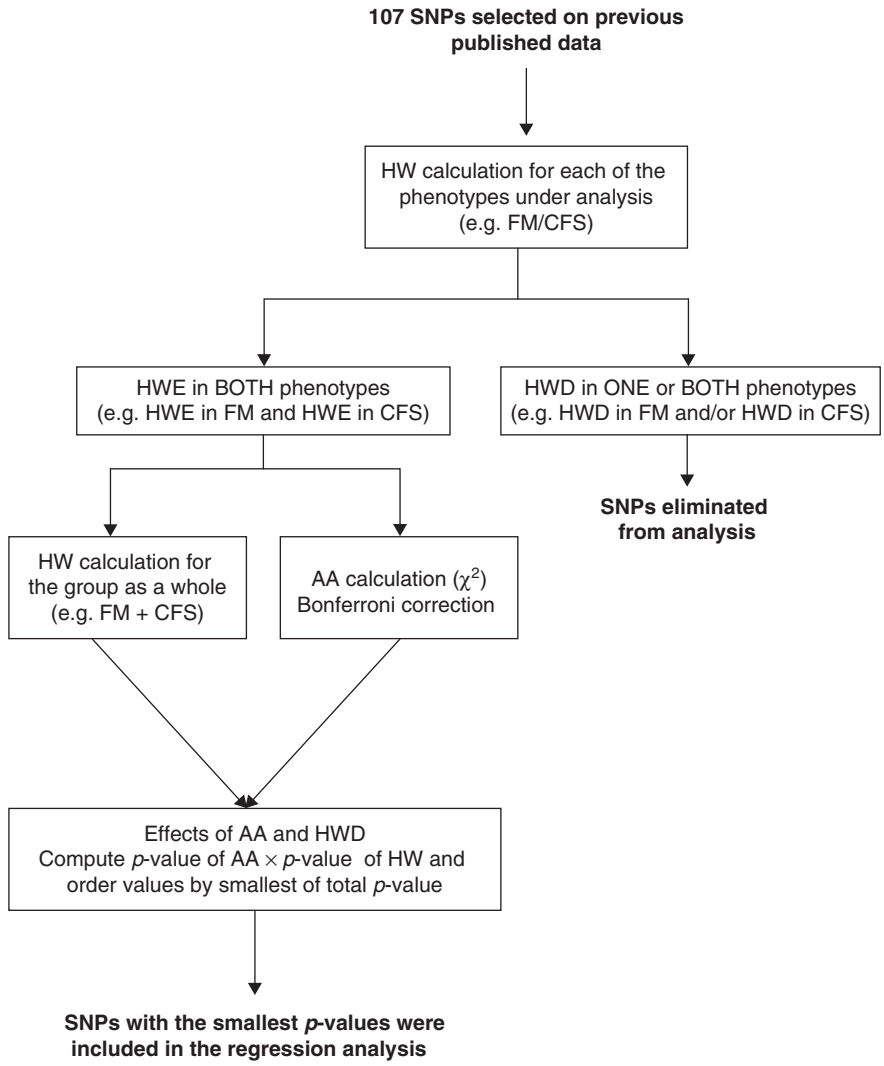
Table 1. SNPs genotyped, their associated genes, chromosomal location and function and allelic associations (continued).

Gene name	*Chromosomal location*	*Gene function*	*Gene symbol*	*SNP number*	χ^2 *p-value*		
					FM vs. CFS/ME	*FM: FIQ >76*	*CFS/ME: CSI >84*
Proopiomelanocortin	2p23.3	Hormone activity	*POMC*	rs6713532	0.000	0.002	0.760
				rs934778	0.164	0.295	0.004
				rs12473543	0.553	0.013	0.000
				rs1866146	0.741	0.153	0.158
Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	17q11.1–q12	Serotonin and monoamine transporter activity	*SLC6A4*	rs2020942	0.000	0.058	0.000
				rs3794808	0.000	0.000	0.041
				rs140700	0.000	0.000	0.301
				rs2228673	0.002	0.787	0.002
				rs7224199	0.004	0.000	0.894
Tumour necrosis factor	6p21.3	Cytokine activity	*TNF*	rs1800629	0.001	0.041	0.256
				rs1800610	0.002	0.643	0.429
				rs3093661	0.752	0.232	0.665
Tryptophan hydroxylase 1	11p15.3–p14	Tryptophan 5-monooxygenase activity	*TPH1*	rs652458	0.000	0.781	0.001
				rs623580	0.007	0.023	0.293
				rs211102	0.655	0.787	0.884
				rs10488682	0.749	0.000	0.000
Tryptophan hydroxylase 2	12q21.1	Tryptophan 5-monooxygenase activity	*TPH2*	rs4760750	0.190	0.818	0.901
				rs1386486	0.717	0.488	0.077
				rs1487280	0.791	0.601	0.004
Thioredoxin reductase 2	22q11.21	Thioredoxin disulfide reductase activity	*TXNRD2*	rs5746847	0.000	0.293	0.211

SNP, single nucleotide polymorphism; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; FIQ, Fibromyalgia Impact Questionnaire; CSI, US Centers for Disease Control and Prevention Symptom Inventory Short Form, Case Definition Score; Chr., chromosome.

Figure 1. Flow diagram illustrating the SNPs selected for the regression analysis.



SNP, single nucleotide polymorphism; HW, Hardy–Weinberg; FM, fibromyalgia; CFS, chronic fatigue syndrome; HWE, Hardy–Weinberg expectation; HWD, Hardy–Weinberg disequilibrium; AA, allelic association.

All genetic analyses were carried out using HelixTree® software (Golden Helix, Inc., Bozeman, MT).

Population stratification in the whole patient group was tested using Partition software (http://www.genetix.univ-montp2.fr/partition/partition.htm)[40].

Statistical modelling

The aim was to obtain a model that could predict which of the two diseases (FM or CFS) a person is likely to exhibit given certain SNP information and, within each disease, to obtain a model that could predict the impact of the disease on the patient's life. Three different models were evaluated: one model to predict a profile to attempt discrimination between FM and CFS/ME (Model 1); one model to predict FM severity (Model 2) (FIQ >76 vs. FIQ ≤76); and a final model (Model 3) to predict CFS/ME severity (CSI >84 vs. CSI ≤84).

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) version 14.0.

Multiple genotype–phenotype associations were analysed by means of multivariate logistic regression (backward logistic regression) with clinically determined disease phenotypes as dependent variables and the individual loci as independent variables[41]. The goodness of fit of the models was evaluated using Hosmer–Lemeshow statistics and their accuracy was assessed by calculating the area under the curve (AUC) of the receiver operating characteristic curve (ROC) with 95% confidence intervals[42]. The explained variability of the models on the basis of the SNPs was evaluated by means of the R^2 Nagelkerke. To measure the impact of the SNPs included in the models of the analysed phenotypes, the sensitivity, specificity and positive likelihood ratio (LR+ = sensitivity/[1–specificity]) were computed by means of ROC curves. Comparisons of mean probability function values between each of the compared phenotypes were performed using a *t*-test. The threshold for statistical significance was predefined as a *p*-level of 0.05.

Haplotype analysis

Haplotype trend regression analysis was carried out and compared with single-locus allelic associations (χ^2 tests) using HelixTree® software.

Results

The presence of population substructure in any genotype–phenotype association study can severely jeopardise its power and efficiency by generating false associations. In this study, the authors recruited patients from all over the country to ensure a homogeneous distribution of the sample population. Analysis of stratification with Partition software confirmed a panmictic population (data not shown).

To differentiate between FM and CFS/ME (Model 1), 20 predictor SNPs were entered into the backward logistic regression model. In stepwise methods, if a predictor does not make a statistically significant

contribution to how well the model predicts the outcome variable, it is removed from the model and the model is re-estimated for the remaining predictors[38]. Therefore, 5 SNPs were excluded and the model based on the remaining 15 fitted the data well (p=0.897, Hosmer–Lemeshow statistic). For Model 2 (FIQ >76 vs. FIQ ≤76), 13 predictor SNPs were entered in the model and 8 were retained (p=0.947, Hosmer–Lemeshow statistic). In Model 3 (CSI >84 vs. CSI ≤84), only 6 of the 11 SNPs initially included in the model remained (p=0.763, Hosmer–Lemeshow statistic). Information regarding the SNPs remaining in each function is shown in Table 2. The contribution of genetic factors to FM and CFS/ME can be further demonstrated by the substantial proportion of variance (R^2 Nagelkerke) explained by genetic factors (57.2% for Model 1, 59.5% for Model 2 and 52.7% for Model 3).

Probability functions were obtained for each phenotype analysed and are presented as box plots in Figure 2. Comparing mean probability function values, statistically significant differences (p<0.001) were found between all subgroups. The sensitivity, specificity and LR+ of all the models are given in Table 3.

Haplotype regression analyses for various marker combinations included in the models were estimated for each of the phenotypes separately. Table 4 displays the results of the overall haplotype association to the disease (via regression analysis) and compares it with the association of each individual locus (via χ^2 tests).

To confirm the validity of the three models described above, a second independent study (validation study) was carried out. Both probability functions (Figure 2) and ROC curves (Table 3) were obtained. A comparison of the ROC–AUCs[41] of the first study (Table 3) and the validation study revealed no significant difference between the two models.

Discussion

Although significant differences in the prevalence of FM and CFS/ME (2–4% for FM and 0.2–0.5% for CFS/ME) have been reported in almost all studies, many publications suggest an important overlap (40–60%) between the two syndromes[43,44], indicating contradictions in the interpretation of the data.

The definition criteria for cases of FM and CFS/ME put particular emphasis on the severity of the symptoms to perform a diagnosis, instead of defining the diseases by their characteristics. Thus, in FM widespread pain and sensitivity to pressure on specific tender points will lead to the diagnosis[19], whereas in CFS/ME major symptoms will be abnormal levels of physical and cognitive fatigue, with a high impact on the pre-morbid activities of the patient[20].

In this study it was noted that 70–80% of the patients with CFS/ME also fulfil the 1990 ACR criteria for FM, but only a small fraction of FM patients overlap symptoms with CFS/ME. Therefore, it was decided that any patient fulfilling the 1990 ACR and the CDC research definition for CFS[20]

Table 2. Variables included in the probability functions and their genotype frequency among the patients included in the study*.

Marker	*Chr.*	*Gene*	*Region*	*Genotype distributions (%)*†					
Discriminating SNPs for FM vs. CFS/ME				*FM*			*CFS/ME*		
				AA	*AB*	*BB*	*AA*	*AB*	*BB*
rs6713532	2	*POMC*	Intron	3	21	76	10	35	55
rs10194776	2	*HTR2B*	Intron	45	48	8	32	48	21
rs1549339	2	*HTR2B*	Intron	49	39	12	58	32	10
rs2168631	5	*DRD1*	5Upstream	5	33	62	4	20	77
rs2229094	6	*LTA*	Coding	57	35	9	41	50	10
rs1800797	7	*IL6*	Intron	24	50	26	13	39	48
rs2770296	13	*HTR2A*	Intron	5	40	55	15	40	45
rs2020942	17	*SLC6A4*	Intron	8	45	47	26	45	29
rs3794808	17	*SLC6A4*	Intron	6	44	50	19	53	29
rs2297518	17	*NOS2A*	Coding	6	38	57	3	30	68
rs5746847	22	*TXNRD2*	Intron	38	47	16	23	42	36
rs933271	22	*COMT*	Intron	44	50	7	63	36	2
rs4680	22	*COMT*	Coding	14	49	37	30	52	19
rs165815	22	*ARVCF*	Coding	4	34	62	2	15	83
rs165774	22	*COMT*	Intron	12	50	38	5	43	52
Discriminating SNPs for FM severity				*FIQ >76*			*FIQ ≤76*		
				AA	*AB*	*BB*	*AA*	*AB*	*BB*
rs10194776	2	*HTR2B*	Intron	58	40	2	38	51	11
rs6713532	2	*POMC*	Intron	0	14	86	4	23	73
rs324029	3	*DRD3*	Intron	24	44	32	6	43	51
rs11246226	11	*DRD4*	3Downstream	0	42	58	25	46	30
rs7224199	17	*SLC6A4*	3Downstream	3	50	48	29	56	15
rs3794808	17	*SLC6A4*	Intron	0	32	68	9	54	37
rs165774	22	*COMT*	Intron	17	57	27	10	47	43
rs4680	22	*COMT*	Coding	6	49	45	20	49	31

Chr., chromosome; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; SNP, single nucleotide polymorphism; FIQ, Fibromyalgia Impact Questionnaire value; CSI, US Centers for Disease Control and Prevention Symptom Inventory Short Form, Case Definition Score.

* Each of these SNPs on their own do not provide accurate differentiation (although some of them do to an extent). They must be used in combination to be a useful predictor.

† 'A' is always the first described allele when a search is made under the rs number in the SNP database from the National Center for Biotechnology Information as of 11 January 2007. For example, rs6713532 is described as C/T, so that A=C and B=T.

Table 2. Variables included in the probability functions and their genotype frequency among the patients included in the study* (continued).

Marker	*Chr.*	*Gene*	*Region*	*Genotype distributions (%)*[†]					
Discriminating SNPs for CFS/ME severity				*CSI >84*			*CSI ≤84*		
				AA	*AB*	*BB*	*AA*	*AB*	*BB*
rs10488682	11	*TPH1*	5Upstream	52	36	13	17	40	43
rs11246226	11	*DRD4*	3Downstream	16	29	55	39	43	19
rs2020942	17	*SLC6A4*	Intron	23	58	19	61	32	8
rs1474347	7	*IL6*	Intron	52	42	7	22	46	32
rs2284217	7	*CRHR2*	Intron	32	52	16	65	30	5
rs489736	X	*HTR2C*	Intron	23	68	10	29	48	23

Chr., chromosome; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; SNP, single nucleotide polymorphism; FIQ, Fibromyalgia Impact Questionnaire value; CSI, US Centers for Disease Control and Prevention Symptom Inventory Short Form, Case Definition Score.

* Each of these SNPs on their own do not provide accurate differentiation (although some of them do to an extent). They must be used in combination to be a useful predictor.

† 'A' is always the first described allele when a search is made under the rs number in the SNP database from the National Center for Biotechnology Information as of 11 January 2007. For example, rs6713532 is described as C/T, so that A=C and B=T.

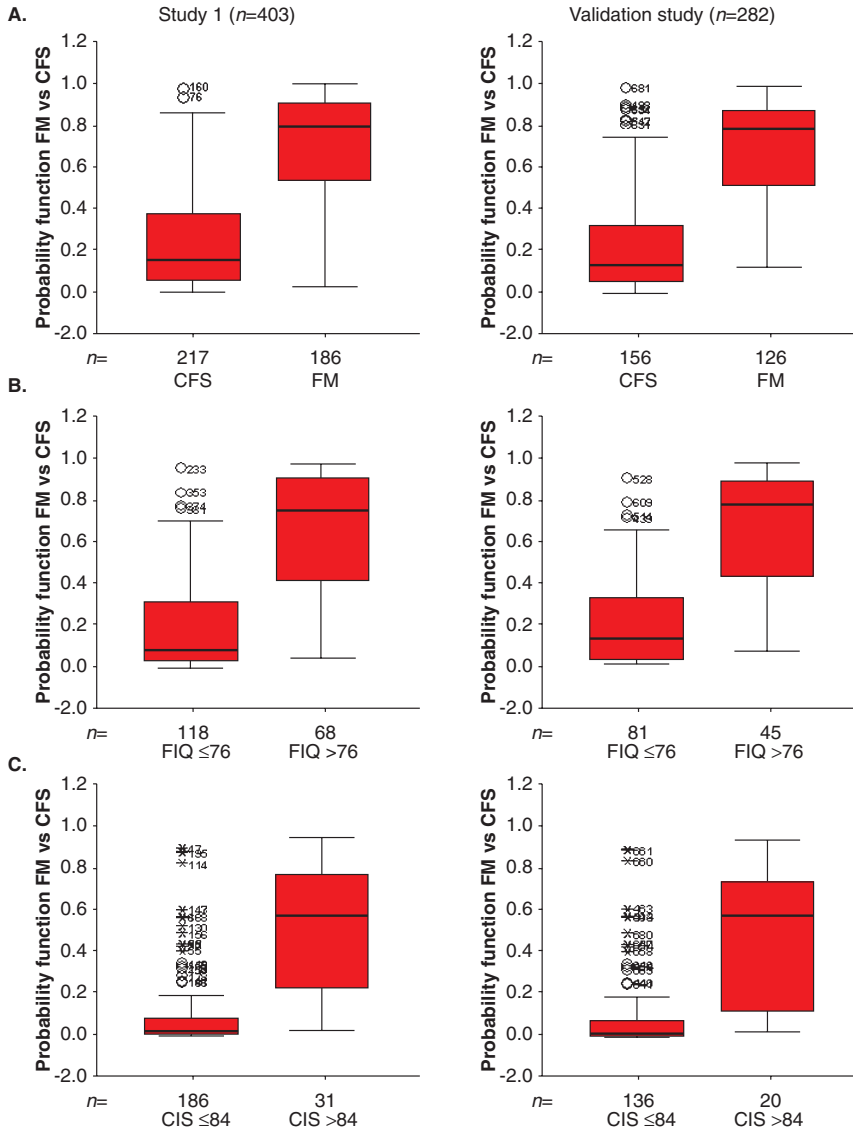
diagnosis criteria would be included in the CFS/ME group, believing that CFS/ME diagnosis should be an exclusion criterion for FM diagnosis. A similar approach has been used in the EULAR evidence-based recommendations for the management of FM syndrome[22].

With this in mind, an important set of SNPs were analysed and those that gave the best models were identified to discriminate with a high LR+ between FM and CFS/ME patients and the severity within each group. Likelihood ratios are a useful and practical way of expressing the power of diagnostic tests[44,45]. In the three models, LR+ ratios >10 were obtained, evidence of the capacity of the SNP combinations to predict each phenotype. The high ROC–AUCs obtained for all the models (>0.89) provide further evidence for the high discriminatory power of the SNP combinations. The usefulness of the ROC–AUC magnitude as a tool for evaluating the strength of the relationship between genotypes and disease has been described previously[46].

The genotypic frequencies presented in Table 2 agree with those already published in the literature. For example, genotype frequencies for rs933271 in the whole fatigued group (63% AA, 36% AB, 2% BB) and for rs2284217 within the CFS/ME CSI ≤84 group (65% AA, 30% AB, 5% BB) are comparable with the ones reported by Goertzel *et al* (49% AA, 51% AB, 0% BB and 58% AA, 40% AB, 2% BB, respectively) in CFS/ME patients[31].

From the SNPs analysed, a combination of 15 were found that could very efficiently discriminate between FM and CFS/ME patients (Table 2). Many

Figure 2. Probability functions presented as box whisker plots. Boxes represent the interquartile range and whiskers are lines that extend from the box to the highest and lowest values. A line across the box indicates the median. Outliers and extreme values are represented with ° and *, respectively. The figures on the left show the box plots for the probability function of the first study and those on the right show the box plots for the validation study. Probability functions for (A) differential diagnosis between patients with FM and CFS/ME, (B) FM syndrome prognosis and (C) CFS/ME prognosis.



FIQ, Fibromyalgia Impact Questionnaire; CSI, US Centers for Disease Control and Prevention Symptom Inventory; CIS, Checklist Invividual Strength; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis.

Table 3. Sensitivity, specificity and positive likelihood ratio (LR+ = sensitivity/[1–specificity]) for the DDx model and the FM and CFS/ME prognosis models computed by means of receiver operating characteristic curves both for the first study and the validation study.

	Study 1 (n=403)				*Validation study (n=282)*				*p-value*
	Sensitivity (%)	*Specificity (%)*	*LR+*	*ROC–AUCs*	*Sensitivity (%)*	*Specificity (%)*	*LR+*	*ROC–AUCs*	
DDx (FM vs. CFS/ME)	52.7	95.4	11.5	0.893	55.6	95.5	12.4	0.913	n.s.
Severity of FM (FIQ >76 vs. FIQ ≤76)	63.2	94.9	12.4	0.906	62.2	95.1	12.7	0.894	n.s.
Severity of CFS/ME (CSI >84 vs. CSI ≤84)	58.1	94.9	12.4	0.912	55.0	95.6	12.5	0.894	n.s.

DDx, differential diagnosis; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; ROC–AUC, receiver operating characteristic curve–area under the curve; FIQ, Fibromyalgia Impact Questionnaire value; CSI, US Centers for Disease Control and Prevention Symptom Inventory Short Form, Case Definition Score; n.s., non-statistical difference between Study 1 and the Validation Study ROC–AUCs models.

of the genes containing those SNPs have been implicated in previous gene studies either with CFS/ME and/or FM. For example, a recently published review[30] pointed out the importance of DRD4, SLC6A4, its receptor and the promoter region in the genetics of FM, and the involvement of *COMT* in pain sensitivity in humans. Also, in 2006 Goertzel *et al* reported polymorphisms belonging to the genes *COMT*, *POMC* and *SLC6A4* among others that could predict whether a person has CFS/ME[31]. In that study, particular importance was given to SNPs from NR3C1 that are not found in these models (Table 2). Among the 107 SNPs analysed, rs6196 and rs852977 had a significant ($p<0.001$ and $p=0.018$, respectively) allelic association between FM and CFS/ME patients and could have been included in the predictor model. However, since other SNPs had a more powerful association and no more than 20 predictors could be included in the regression analysis[37], they were excluded from the analysis.

It is also important to consider that more than 50% of the variation in the population was explained by genetic factors (57.2% for Model 1, 59.5% for Model 2 and 52.7% for Model 3). This fact agrees with previous family-based studies[30] that found a higher prevalence of FM and/or CFS/ME among family members, stressing the importance of genetic factors in the aetiology of those pathologies.

Stratification of the symptoms forms part of the diagnosis of both diseases and it is necessary for a correct therapeutic and

Table 4. Haplotype association analysis and individual single nucleotide polymorphism associations.

Chr.	*Haplotype*	*χ^2 p-value*	*Bon. p-value**
		FM vs. CFS/ME	
2	Hap. 1 (rs6713532, rs10194776, rs1549339)	4.7 E-11	1.9 E-10
	rs6713532	1.5 E-05	3.3 E-04
	rs10194776	1.2 E-04	2.5 E-03
	rs1549339	8.2 E-02	8.8 E-01
17	Hap. 2 (rs2020942, rs3794808, rs2297518)	3.0 E-13	1.2 E-12
	rs3794808	4.1 E-07	1.6 E-06
	rs2020942	9.1 E-07	2.3 E-05
	rs2297518	2.0 E-02	2.6 E-01
22	Hap. 3 (rs5746847, rs933271, rs165774, rs4680, rs165815)	1.2 E-13	7.1 E-13
	rs165815	2.9 E-06	1.1 E-04
	rs5746847	4.3 E-06	1.6 E-04
	rs4680	4.2 E-05	1.4 E-03
	rs933271	1.2 E-04	3.7 E-03
	rs165774	6.8 E-03	1.5 E-01
		FM: FIQ >76 vs. FIQ ≤76	
2	Hap. 4 (rs6713532, rs10194776)	5.0 E-04	1.5 E-03
	rs10194776	3.0 E-03	2.2 E-02
	rs6713532	2.2 E-02	1.7 E-01
17	Hap. 5 (rs7224199, rs3794808)	1.5 E-14	4.4 E-14
	rs7224199	4.8 E-09	1.4 E-08
	rs3794808	1.4 E-05	9.7 E-05
22	Hap. 6 (rs165774, rs4680)	1.4 E-06	4.3 E-06
	rs165774	4.8 E-03	3.6 E-02
	rs4680	1.6 E-02	1.2 E-01
		CFS: CSI >84 vs. CSI ≤84	
7	Hap. 7 (rs2284217, rs1474347)	1.8 E-08	5.5 E-08
	rs1474347	1.4 E-05	9.7 E-05
	rs2284217	4.9 E-04	3.7 E-03
11	Hap. 8 (rs10488682, rs11246226)	1.7 E-05	1.7 E-05
	rs10488682	6.1 E-05	6.1 E-05
	rs11246226	4.4 E-03	4.4 E-03

Chr., chromosome; FM, fibromyalgia; CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; Hap., haplotype; FIQ, Fibromyalgia Impact Questionnaire value; CSI, US Centers for Disease Control and Prevention Symptom Inventory Short Form, Case Definition Score.
* *p*-value after correction with Bonferroni for multiple testing.

prognostic orientation. The definition of disease subtypes using self-referring tests requires underpinning with biological data[10]. Therefore, identification of well defined clinical subgroups, by means of biological markers, is the next logical step in the investigation of FM and CFS/ME. Up-to-date self-referring questionnaires have been successfully used to assess the severity of the diseases. The relationship between FIQ values, the severity of disease and the degree of disability have been reported on a number of occasions[47,48]. Performance of this test (FIQ) is also recommended in disability claims for evaluation of the degree of impairment caused by the disease[49].

The models described in this paper are also suitable for differentiating between severe and milder phenotypes of these diseases in a female Spanish population. An attempt to differentiate between subgroups in CFS/ME patients using genetic analysis has previously been reported in the literature[11]. In agreement with that study, no significant associations were found in CFS/ME subtypes within the *COMT* gene or in rs1800795, rs7209436, rs173365 and rs2267710 (data not shown), whereas rs12473543 was considered significantly associated ($p<0.001$). However, the results with other important markers (mainly related to MAOA, MAOB and NR3C1 polymorphisms) differ. Whilst Smith *et al* tried to identify many CFS/ME subgroups based on different symptoms[11], the authors tried to demonstrate that the impact of the disease on the patient's quality of life (as assessed by validated questionnaires) was in part dependent on the genetic profile. These models (Table 3) not only validate the usefulness of the self-referring questionnaires for the stratification of FM and CFS/ME, but also demonstrate for the first time the great differences in the genetic components of the two diseases.

To test further the validity of the SNPs, haplotype analysis was carried out. In all cases the haplotypes were much more significant than single-locus associations, which highlights the loss of information when only one SNP is taken into account as well as the fact that SNP combinations give more powerful models.

Conclusions

To the authors' knowledge, they are the first to carry out a study in which both patient groups (CFS and FM) are clearly differentiated and compared. The findings confirm that we are faced with two complex syndromes, of exclusively clinical definition until now. In addition, they have identified genetic differences within each of the pathologies, suggesting the presence of subtypes that correlate with the degree of disability of the disease. These subtypes could represent different illnesses or clinical situations that, in the future, could be differentiated.

The existence of a distinctive genetic profile related to the patients most impaired by the diseases can help us to understand its predisposing, precipitating and perpetuating factors and could allow us to ameliorate the symptoms or choose

an appropriate therapeutic approach. The authors are aware that the findings reported here should be validated in other populations with different genetic backgrounds and that the number of SNPs could be increased to obtain a better discrimination between clinical variants of FM and CFS/ME. However, validation of the models in a separate study using a different set of patients makes us very confident of these results, at least for the Caucasian population.

The findings of this work suggest that, at least for a subgroup of women with FM and CFS, distinguishing genetic profiles exist for each illness and these confirm the interest of genomic study in complex disorders, without overlooking the fact that perpetuating trigger factors play a relevant role in them.

These distinguishing profiles and their significance appear to be statistically powerful when the CFS diagnosis is based on objective abnormal fatigue tests of both a physical and cognitive nature. In the authors' opinion, a more subjective diagnosis can greatly alter these results. The possible slant induced by subjectivity in quality of life measures, whilst still valid, must also be taken into account.

One important limitation of this study is the lack of comparison with a control group. The research group is developing this aspect at the time of publication.

However, this methodology is still dependent on a preliminary reliable diagnosis that fulfils all the disease inclusion and exclusion criteria, which might still be under debate. The authors hope that these results could lead to more research into a previously unexplored area and that the information could improve the quality of other scientific work on these two pathologies.

Validation of the findings would consolidate the authors' proposal that CFS must be considered, in this development stage of research, as an exclusion diagnosis for FM diagnosis.

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Conflicts of interests: Progenika Biopharma, S.A. requested a patent for the findings of this study (application number GB 061 3842.2).

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