Microbial infections in eight genomic subtypes of chronic fatigue syndrome/myalgic encephalomyelitis

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ABSTRACT

Background The authors have previously reported genomic subtypes of chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) based on expression of 88 human genes.

Aim To attempt to reproduce these findings, determine the specificity of this signature to CFS/ME, and test for associations between CFS/ME subtype and infection.

Methods Expression levels of 88 human genes were determined in blood of 62 new patients with idiopathic CFS/ME (according to Fukuda criteria), six patients with Q-fever-associated CFS/ME from the Birmingham Q-fever outbreak (according to Fukuda criteria), 14 patients with endogenous depression (according to DSM-IV criteria) and 29 normal blood donors.

Results In patients with CFS/ME, differential expression was confirmed for all 88 genes. Q-CFS/ME had similar patterns of gene expression to idiopathic CFS/ME. Gene expression in patients with endogenous depression was similar to that in the normal controls, except for upregulation of five genes (*APP*, *CREBBP*, *GNAS*, *PDCD2* and *PDCD6*). Clustering of combined gene data in CFS/ME patients for this and the authors' previous study (117 CFS/ME patients) revealed genomic subtypes with distinct differences in SF36 scores, clinical phenotypes, severity and geographical distribution. Antibody testing for Epstein–Barr virus, enterovirus, *Coxiella burnetii* and parvovirus B19 revealed evidence of subtype-specific relationships for Epstein–Barr virus and enterovirus, the two most common infectious triggers of CFS/ME.

Conclusions This study confirms the involvement of these genes in CFS/ME.

INTRODUCTION

Chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) is a disease characterised by severe and debilitating fatigue, sleep abnormalities, impaired memory and concentration, and musculoskeletal pain.[1] In the Western world, the population prevalence is estimated to be of the order of 0.5%.[2 3] Research studies have identified various features relevant to the pathogenesis of CFS/ME such as viral infection, immune abnormalities and immune activation, exposure to toxins, chemicals and pesticides, stress, hypotension, lymphocyte abnormalities and neuroendocrine dysfunction. However, the precise underlying disease mechanisms and means by which these abnormalities inter-relate in patients with CFS/ME remain to be clarified.[4 5]

Various groups have analysed the gene expression in peripheral blood of patients with CFS/ME, and, in all of these studies, genes of immunity and defence are prominent. Following a pilot microarray study which identified 16 abnormally expressed genes in CFS/ME,[6] we reported on a comprehensive microarray study which reveals abnormal expression of 88 human genes in patients with CFS/ME.[7] Clustering of these data revealed seven genomic subtypes of CFS/ME with distinct differences in SF36 scores, clinical phenotypes, severity and geographical distribution.[7 8] However, remaining questions relate to reproducibility and the specificity of these gene abnormalities to CFS/ME and possible associations with infectious agents.

In this study, we set out to determine whether these findings were reproducible in fresh subjects, whether the previously reported dysregulation of these genes also occurred in drug-free patients with endogenous depression, and whether there was any relationship between particular microbial infections and CFS/ME genomic subtype. The results show that these findings are reproducible and that gene expression in patients with endogenous depression was markedly different from that in patients with CFS/ME, and was similar to that in the normal controls, in terms of these 88 human genes. Also, clustering of gene data revealed eight genomic subtypes with distinct clinical differences, and several of these had interesting associations with particular microbial infections.

METHODS

Subject enrolment, clinical characterisation and blood sampling

Patients with CFS/ME (n=62), who lived in Birmingham (n=6), Bristol (n=3), London (n=9) and New York (n=44), were diagnosed according to Fukuda diagnostic criteria for CFS/ME[1] and enrolled into the study. All had idiopathic CFS/ME except the six Birmingham patients, who had CFS/ME that had been triggered by laboratory-documented Q fever. Patients with psychiatric disease were excluded using the Minnesota International Neuropsychiatric Interview, thus ensuring that none of our CFS/ME patients had major psychiatric disease or misused alcohol or other drugs. Clinical and quantitative PCR (qPCR) data for these new patients were combined with those for 55 CFS/ME patients from a previous study,[7 8] giving a total of 117 CFS/ME patients, who lived in Birmingham (n=6), Bristol (n=14), Leicester (n=1), London (n=12), New York (n=55) and Dorset (n=28).

Patients with endogenous depression (n=14) were enrolled from Bristol, UK, and surrounding area. These patients fulfilled *Diagnostic and statistical manual of mental disorders*, 4th edition (DSM-IV) criteria, had not

smoked within the previous year, and had not taken antidepressants in the previous year.

Healthy normal blood donors enrolled from the Dorset National Blood Service (n=29) were used as a comparison group. Restrictions imposed by the Dorset National Blood Service on those allowed to donate blood are outlined elsewhere.[6]

For all patient groups, individuals who smoked in the previous year, who abused alcohol or other drugs, or were currently taking (or were within 3 months of taking) antibiotics, steroids, cytotoxic drugs or antidepressants were excluded from the study.

For all enrolled subjects (patients and controls), according to the recommendations of the International CFS Study Group,[9] severity of physical and mental fatigue was assessed using the Chalder Fatigue Scale,[10] level of disability was assessed using the Medical Outcomes Survey Short Form-36 (SF36), accompanying symptoms were characterised using the Somatic and Psychological Health Report, sleep abnormalities were assessed using the Pittsburgh Sleep Questionnaire, and assessment of type and severity of pain was performed using the McGill Pain Questionnaire.

Patients and controls gave informed written consent according to guidance of the Wandsworth Research Ethics Committee (approval number 05/Q0803/137). For the New York patients, approval of the local institutional review board was obtained. The human experimentation guidelines of the US Department of Health and Human Services were followed in this study.

A 2.5 ml sample of blood was taken from both CFS/ME patients and normal blood donors (as part of routine blood donation) into PAXgene tubes (PreAnalytix, Qiagen, UK), and total RNA extracted using the PAXgene blood RNA kit (PreAnalytix), according to the instructions of the manufacturer. RNA quality and amount were confirmed by micro-spectrophotometry (Nanodrop, Rockland, Delaware, USA). Total RNA samples used in this study had an absorbance ratio (A_{260}/A_{280}) of 1.9–2.0.

Quantitative PCR

qPCR (Applied Biosystems, Foster City, California, USA) was used to quantitate the amount of mRNA for 88 CFS/ME-associated human genes by the comparative method, using custom 384-well low-density arrays and the ABI PRISM 7900HT instrument (Applied Biosystems), with glyceraldehyde-3-phosphate dehydrogenase as the endogenous control gene. Experiments were performed in triplicate using the protocol described previously.[6 7] Data were displayed using SDS v2.2 software (ABI), discordant data between replicates were omitted, and results for each low-density array were calculated and loaded into ABI SDS v2.2 Enterprise Edition software.

The threshold cycle (Ct) for each test gene in each sample was compared with that for glyceraldehyde-3-phosphate dehydrogenase to calculate a ΔCt value. ΔCt values were then normalised to the calibrator sample to give the ΔΔCt values. Relative quantities (RQ) ($2^{-\Delta\Delta Ct}$) of each mRNA of interest were then calculated. Samples showing a difference between minimum and maximum RQ values of ≥100 (indicating poor replicate concordance) were excluded. The t test was used to compare mean RQ values between groups. $p \leq 0.05$ was taken to be significant.

Clustering of qPCR-generated gene values of CFS/ME patients

Ct values for all 88 CFS/ME-associated genes in 117 CFS/ME patients were then normalised and clustered using Genesis software.[11] For each of the eight CFS/ME subtypes identified using this approach, mean RQ values were calculated for each gene, and used to generate fold-difference (CFS/ME/normal) values for each gene in each CFS/ME subtype. Mean fold-difference values for each gene in each CFS/ME subtype were then clustered with and without normalisation/median centring using Cluster v2.11 software and visualised using Treeview v1.60 software.[12] The clustering algorithm in both of these software programs has been described previously.[12]

Detection of anti-microbial antibodies

IgM and IgG antibodies specific to four microbes that are well recognised to trigger CFS/ME were detected by ELISA, according to the manufacturer's instructions: Epstein-Barr virus (EBV) (viral capsid antigen (VCA) IgM and IgG, early antigen IgG and Epstein–Barr nuclear antigen (EBNA) IgG; Meridien Bioscience Inc, Cincinnati, Ohio, USA), enterovirus (all serotypes; Virion Serion, Wurzberg, Germany), parvovirus B19 (viral protein 2 IgM and IgG; Biotrin, Dublin, Ireland) and *Coxiella burnetii* (phase I and II IgG; Virion Serion).

Statistical testing

Testing of the significance of associations of gene expression levels with different patient groups was performed using a two-tailed t test. Testing of the significance of association between clinical variables and CFS/ME genomic subtype was performed using χ^2, analysis of variance (ANOVA) and the Mann–Whitney U tests. Testing of the significance of association between microbial markers in CFS/ME and CFS/ME subtypes was performed using χ^2 analysis and ANOVA.

RESULTS

Subjects and clinical characterisation

A total of 117 patients with CFS/ME fulfilling Centers for Disease Control diagnostic criteria were used in this study. For 55, previously published data were used, while the remaining 62 had not previously been tested; for six of these, CFS/ME disease had been triggered by laboratory-documented *C burnetii* infection. In addition, 14 patients with endogenous depression and 29 normal blood donors were studied.

A summary of the clinical details of these subjects is shown in table 1. In general, all CFS/ME groups had similar profiles of symptoms and mean clinical scores, and Q-CFS/ME was phenotypically similar to the other CFS/ME cases in which the triggering factors were unknown. Patients with endogenous depression had a markedly low prevalence of numbness/tingling and tender lymphadenopathy, and less bodily pain, as indicated by the McGill Pain Questionnaire mean score, as compared with CFS/ME. Normal blood donors had very low prevalence of all symptoms, little fatigue (Chalder), pain (McGill), associated symptoms (Somatic and Psychological Health Report), normal sleep (Pittsburgh Sleep Questionnaire Index) and high SF36 total scores (table 1), as would be expected.

Quantitative PCR

qPCR was carried out using TaqMan primers/probes specific for 88 human genes that were previously found to be differentially expressed in CFS/ME patients.[7] This analysis confirmed that most of these genes differed significantly between CFS/ME and the normal group. Of the 88 genes, 84 were found to be upregulated and four were downregulated (*HIF1A, IL7R, PAPOLA, SHPRH*), which is similar to what we reported previously.[7] Gene expression in patients with Q-CFS/ME was also found to be markedly different from the normal group, and very similar to that found in patients with CFS/ME. Gene expression in patients with endogenous depression did not differ markedly from that in the normal group, except in the case of five genes (*APP, CREBBP, GNAS, PDCD2, PDCD6*), where significant upregulation (fold difference ≥1.5) was found (table 2).

Table 1 Patient information including age, sex, symptoms and questionnaire results summarising fatigue severity, pain, sleep, general function and associated symptoms for patients with CFS/ME and normal blood donors enrolled in microarray and real-time PCR studies, respectively

Variable	CFS/ME patients in previous study[7] (n=55)	CFS/ME patients, previously untested (n=56)	Q-CFS/ME patients* (n=6)	All CFS/ME patients (n=117)	Patients with endogenous depression (n=14)	Normal blood donors (n=29)
Gender (M:F)	19:36	10:46	6:0	35:82	4:10	14:15
Mean age (years)	41.6	40.25	41.5	41.3	41.36	44.6
Mean duration of disease (years:months)	3.17	2.9	5.7	3.4	0:6	NA
Symptoms/signs						
Headache	26	30	1	57	5	1
Sore throat	27	29	0	56	1	1
Poor memory/concentration	30	46	4	80	11	3
Muscle pain	37	42	6	85	5	2
Muscle weakness	36	31	5	72	2	1
Joint pain	41	52	6	99	8	1
Post-exertional malaise	47	54	5	106	9	2
Sleep problem	44	24	0	68	4	3
Gastrointestinal problems	35	36	2	73	6	1
Fainting/dizziness	25	45	5	75	8	1
Numbness/tingling	24	25	2	51	1	0
Tender lymphadenopathy	27	22	2	51	0	0
Mean scores						
Physical fatigue (Chalder)	16.13	14.36	10.83	15.15	14.00	7.69
Mental fatigue (Chalder)	8.05	7.34	6.00	7.98	7.42	4.24
McGill Pain Questionnaire	15.28	18.57	18.80	17.58	9.67	2.48
Sphere questionnaire	11.25	11.21	7.33	10.87	12.45	2.07
SF36 questionnaire	46.45	38.65	52.85	45.12	46.19	83.61
Pittsburgh Sleep Quality Index	10.22	10.00	8.17	10.01	12.25	4.28

*These six Q-CFS/ME patients were all part of the 1989 Birmingham Q-CFS/ME outbreak cohort.
CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; NA, not applicable; Sphere, Somatic and Psychological Health Report.

Genomic CFS/ME subtypes

Clustering of ΔCt values for the 88 CFS/ME-associated genes in the 117 CFS/ME patients identified eight subtypes (designated A–H), consisting of 27, 6, 19, 5, 21, 13, 19 and 4 CFS/ME patients, respectively. There were three patients whose gene profile did not fit into any of these eight subtype groupings. Mean fold-difference values for each CFS/ME subtype are shown in table 3 and figure 1. Most genes in each subtype were shown to be upregulated (figure 1 and table 3).

The relationship between the subtypes of the present study and those of the previous study which examined only 55 CFS/ME patients[7 8] may be difficult to determine. As these subtypes are derived by using clustering, which finds similar groups on the basis of gene expression values, there is no means to predict the outcome of the clustering. As there was incomplete preservation of the previous CFS/ME patient groupings in the present study, we have designated the subtypes, A–H, to distinguish them from those of the previous study, which were designated 1–7.[7]

Analysis of sex ratios for each subtype reveals that subtype D is made up of females only, subtype H is made up of equal numbers of males and females, and the remaining subtypes are made up predominantly of females.

It is particularly interesting that five of six CFS/ME patients with Q-CFS/ME clustered in the same subtype (subtype A).

The clinical phenotype was distinct between subtypes. Subtype D was the most severe, having the lowest scores for SF36 modules RP, VIT, GH, BP and total score, and the highest frequency of occurrence of muscle pain and sleep problems (see figure 2 for definitions of abbreviations for SF36 modules). Subtype B was the least severe, having the highest scores for SF36 modules RP, GH, MH and total score. Subtype B had a higher median score for the SF36-RP (physical role) than all the others combined (87.5 vs 0), p=0.04; Mann–Whitney U test). However, subtype B had the highest frequency of cognitive dysfunction, muscle weakness and post-exertional malaise. Subtype B showed a higher frequency of cognitive dysfunction than all non-subtype B patients combined (p=0.03) and showed an increased severity and duration of headache compared with all non-subtype B patients combined (p=0.02). Subtype B also had a higher median score for mental fatigue (Chalder scale) than all non-subtype B patients combined, although this did not reach significance (9.5 vs 7.0; p=0.06). Subtypes B and C had the best mental health scores, and subtypes A and F had the worst (figure 2A,B).

Subtype E had a higher median score for SF36-VIT than all the others combined (35.0 vs 15.0; p=0.05; Mann–Whitney U test). Subtype E resulted in the highest frequency of gastrointestinal problems. Patients of subtype F showed a higher frequency of increased severity of numbness/tingling compared with all non-subtype F patients combined (p=0.03). Patients of subtype H showed an increased frequency of severity of sore throat compared with all non-subtype H patients combined (p=0.01) (figure 2A,B).

As regards possible association of subtype with geographical location, there was evidence to support this, as we found previously.[7] Predominant subtypes in each geographical location were as follows: Birmingham, subtype A; Bristol, subtype C;

Table 2 CFS/ME-associated genes and transcription factors in patients with CFS/ME, Q-fever-associated CFS/ME and endogenous depression

Gene symbol	GenBank accession number	Taqman assay ID‡	CFS/ME (n=111)		Q-CFS/ME (n=6)		Endogenous depression (n=14)	
			Fold difference	p Value	Fold difference	p Value	Fold difference	p Value
*ABCD4**	NM_020323	Hs00245340_m1	2.21	0.01	3.01	0.031	1.42	0.26
ACTR3	NM_005721	Hs00828586_m1	13.53	0.0029	17.77	0.04	1.22	0.72
AKAP10	NM_007202	Hs00183673_m1	5.22	0.01	6.28	0.031	1.16	0.38
*ANAPC11**	NM_016476	Hs00212858_m1	2.57	0.006	1.47	0.002	1.11	0.37
ANAPC5	NM_016237	Hs00212120_m1	2.04	0.002	1.07	0.045	1.32	0.392
APP	NM_201413	Hs00169098_m1	1.42	0.00003	0.23	0.001	1.59	0.021
ARL4C	NM_005737	Hs00255039_s1	7.15	0.00001	5.25	0.0023	1.18	0.76
ARPC5	NM_005717	Hs00271722_m1	3.71	0.000008	4.26	0.0047	1.46	0.49
ARSD	NM_001669	Hs00534692_m1	1.62	0.001	1.65	0.05	1.07	0.133
ATP6V1C1	NM_001695	Hs00184625_m1	2.66	0.0009	2.03	0.021	1.23	0.75
BCOR	NM_017745	Hs00372369_m1	1.90	0.0045	2.37	0.007	1.09	0.28
BMP2K	NM_198892	Hs00214079_m1	8.05	0.01	14.27	0.04	1.18	0.125
*BRMS1**	NM_015399	Hs00363036_m1	3.08	0.0002	2.43	0.037	1.28	0.194
*CD2BP2**	NM_006110	Hs00272036_m1	4.12	0.000084	5.15	0.001	1.44	0.334
CD47	NM_198793	Hs00179953_m1	3.38	0.0007	2.60	0.002	1.07	0.125
CEP350	NM_014810	Hs00402774_m1	3.85	0.001	6.49	0.01	1.36	0.803
CITED2	NM_006079	Hs00366696_m1	5.28	0.000031	6.21	0.049	1.33	0.172
CMTM6	NM_017801	Hs00215083_m1	3.61	0.014	6.31	0.046	1.21	0.405
CREBBP	NM_004380	Hs00231733_m1	7.02	0.02	9.82	0.025	1.61	0.021
CRK	NM_016823	Hs00180418_m1	1.98	0.000044	1.29	0.0003	1.40	0.683
CTBP1	NM_001328	Hs00179922_m1	5.13	0.071	4.16	0.02	1.45	0.134
CXCR4	NM_003467	Hs00607978_s1	13.46	0.00009	28.13	0.007	1.05	0.128
EBI2	NM_004951	Hs00270639_s1	5.99	0.002	26.16	0.011	0.88	0.687
EGR1	NM_001955	Hs00152928_m1	1.69	0.03	0.34	0.026	1.33	0.65
EGR3	NM_004421	Hs00231780_m1	2.11	0.017	–	–	–	–
*EIF2B4**	NM_172195	Hs00248984_m1	2.87	0.0026	1.13	0.048	0.58	0.739
EIF3S10	NM_003750	Hs00186707_m1	2.10	0.0034	1.55	0.067	1.29	0.295
*EIF4G1**	NM_198241	Hs00191933_m1	2.42	0.0007	0.34	0.035	1.16	0.165
EIF4G3	NM_003760	Hs00186804_m1	2.17	0.00012	3.22	0.0079	1.35	0.83
ETS1	NM_005238	Hs00901425_m1	30.82	0.0008	37.57	0.055	1.09	0.761
FAM126B	NM_173822	Hs00545158_m1	3.19	0.01	5.52	0.03	1.26	0.906
FNTA	NM_002027	Hs00357739_m1	3.86	0.0007	2.80	0.001	1.24	0.254
*GABARAPL1**	NM_031412	Hs00744468_s1	5.27	0.00042	1.83	0.008	1.47	0.525
GABPA	NM_002031	Hs00745591_s1	15.40	0.0001	1.91	0.027	1.12	0.716
GCN1L1	NM_006836	Hs00412445_m1	1.18	0.00072	0.70	0.0015	1.07	0.443
GLTSCR2	NM_015710	Hs00414236_m1	5.49	0.0016	5.03	0.038	1.17	0.807
GNAS	NM_080425	Hs00255603_m1	2.37	0.000045	1.56	0.0021	1.56	0.004
*GSN**	NM_198252	Hs00609276_m1	2.56	0.00037	2.40	0.01	1.11	0.312
GTF2A2	NM_004492	Hs00362112_m1	1.08	0.002	0.52	0.039	1.13	0.375
HIF1A	NM_001530	Hs00153153_m1	0.66	0.019	2.67	0.012	1.28	0.255
IFNAR1	NM_000629	Hs00265057_m1	3.30	0.00025	3.02	0.009	1.32	0.853
*IL10RA**	NM_001558	Hs00387004_m1	1.34	9.87E-06	–	–	–	–
IL6R	NM_000565	Hs00794121_m1	2.49	0.06	–	–	–	–
IL6ST	NM_002184	Hs00174360_m1	3.34	0.0011	1.67	0.034	1.36	0.617
IL7R	NM_002185	Hs00233682_m1	0.52	0.032	–	–	–	–
JAK1	NM_002227	Hs00233820_m1	12.73	0.0000008	15.51	0.04	1.05	0.623
*KHSRP**	NM_003685	Hs00269352_m1	1.82	0.00026	0.35	0.0016	1.22	0.55
MAPK9	NM_139070	Hs00177102_m1	1.58	0.045	1.29	0.05	0.95	0.213
METTL3	NM_019852	Hs00219820_m1	1.30	0.0001	0.77	0.01	1.17	0.215
*MRPL23**	NM_021134	Hs00221699_m1	2.62	0.001	0.80	0.029	1.36	0.79
MRPS6	NM_032476	Hs00606808_m1	2.75	0.025	1.87	0.014	1.34	0.451
MRRF	NM_138777	Hs00751845_s1	8.23	0.0004	2.84	0.03	1.22	0.25
MSN†	NM_002444	Hs00792607_mH	4.85	0.0016	7.49	0.002	1.35	0.962
MTMR6	NM_004685	Hs00395064_m1	6.60	0.0025	4.12	0.048	1.12	0.15
NFKB1	NM_003998	Hs00231653_m1	5.01	0.00027	5.01	0.001	1.28	0.41
NHLH1	NM_005589	Hs00271582_s1	58.31	7.00E-04	–	–	–	–
NR1D2	NM_005126	Hs00233309_m1	2.06	0.00016	1.56	0.0006	0.73	0.96
*NTE**	NM_006702	Hs00198648_m1	2.92	0.001	7.34	0.02	1.49	0.579
NUFIP2	NM_020772	Hs00325168_m1	2.37	0.001	2.00	0.046	1.31	0.929
PAPOLA	NM_032632	Hs00413685_m1	0.62	0.00021	0.45	0.001	1.37	0.672

Continued

Table 2 Continued

Gene symbol	GenBank accession number	Taqman assay ID‡	CFS/ME (n=111)		Q-CFS/ME (n=6)		Endogenous depression (n=14)	
			Fold difference	p Value	Fold difference	p Value	Fold difference	p Value
*PDCD2**	NM_002598	Hs00751277_sH	5.38	0.008	–	–	1.62	0.029
PDCD6	NM_013232	Hs00737034_m1	2.54	0.0002	2.19	0.01	1.69	0.015
*PEX16**	NM_004813	Hs00191337_m1	3.98	0.0061	3.32	0.028	0.68	0.776
PGM2	NM_018290	Hs00217619_m1	4.28	0.000001	3.50	0.0014	1.07	0.308
PIK3R1	NM_181523	Hs00236128_m1	4.04	0.005	2.60	0.01	1.14	0.208
*PKN1**	NM_213560	Hs00177028_m1	4.58	0.0003	3.95	0.01	1.03	0.887
*POLR2G**	NM_002696	Hs00275738_m1	2.71	0.001	1.00	0.039	0.77	0.916
PPP2R5C	NM_002719	Hs00604902_m1	4.65	0.013	8.21	0.045	1.30	0.906
PRKAA1	NM_006251	Hs01562315_m1	4.19	0.0002	2.18	0.001	1.29	0.56
PRKAR1A	NM_002734	Hs00267597_m1	3.55	0.0000004	2.31	0.0001	1.23	0.83
PUM2	NM_015317	Hs00209677_m1	2.73	0.00078	2.33	0.002	1.35	0.82
RAP2C	NM_021183	Hs00221801_m1	6.74	0.013	4.37	0.043	1.46	0.69
REPIN1	NM_013400	Hs00274221_s1	4.51	0.00001	2.13	0.01	1.26	0.41
RNF141	NM_16422	Hs00212656_m1	6.49	0.00000079	7.44	0.0003	1.19	0.411
SELENBP1	NM_003944	Hs00187625_m1	10.57	0.001	7.00	0.02	1.06	0.104
SFXN1	NM_022754	Hs00224259_m1	1.69	0.041	0.69	0.037	1.00	0.24
SHPRH	NM_173082	Hs00542737_m1	0.56	0.02	0.69	0.03	1.00	0.303
SNAP23	NM_003825	Hs00187075_m1	7.00	0.0006	3.17	0.01	1.10	0.132
SORL1	NM_003105	Hs00268342_m1	1.67	4.10E-08	–	–	–	–
SOS1	NM_005633	Hs00362308_m1	1.02	0.001	1.02	0.037	1.27	0.52
TAF11	NM_005643	Hs00194573_m1	1.17	0.001	0.00	0.02	1.40	0.57
TCF3	NM_003200	Hs00413032_m1	2.40	0.03	1.63	0.068	1.29	0.86
TDP1	NM_018319	Hs00217832_m1	3.12	0.001	3.16	0.01	1.12	0.83
TNFRSF1A	NM_001065	Hs00533560_m1	12.37	0.004	18.52	0.03	0.86	0.279
UBTF	NM_014233	Hs00610729_g1	6.38	0.002	2.40	0.011	1.09	0.297
USP38	NM_032557	Hs00261419_m1	3.35	0.01	4.98	0.078	1.43	0.367
WAPAL	NM_015045	Hs00386162_m1	3.94	0.003	3.69	0.026	1.17	0.44
WDR26	NM_025160	Hs00228535_m1	1.36	0.0008	0.71	0.01	1.48	0.95

*Genes found in pilot study.[13]
†Genes found in study using differential display/PCR.[7]
‡Taqman assays were those pre-designed by Applied Biosystems, Warrington, UK.
CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis.

Leicester, subtype C; London, subtype C, then subtype G; New York, subtype E, then subtypes G, A, C and F; Dorset, subtypes A, F, B. Subtype A was prominent in New York, Birmingham and Dorset; subtype B was prominent in Dorset; subtype C was prominent in Bristol, London and New York; subtype D was prominent in Bristol and London; subtype E was prominent in New York; subtype F was prominent in Dorset and New York; subtype G was prominent in New York; subtype H was prominent in Dorset (figure 2C).

Microbial infections

The presence and titre of specific antibodies (IgM and IgG) to four treatable microbial infections that are well recognised as triggers of CFS/ME were also determined in serum samples; these were EBV, enterovirus, parvovirus B19 and *C burnetii*. The seroprevalence (proportion of subjects who were positive for specific IgG) of each of these infections was typical of the general population: EBV (based on VCA IgG), 88%; enterovirus, 49%; parvovirus B19 (based on viral protein 2 IgG), 74%; *C burnetii* (based on phase I or II IgG), 10%. Of the 11 patients who had *C burnetii* IgG, five were patients whose CFS/ME disease had been triggered by laboratory-documented Q fever.

CFS/ME patients with acute infection with one or more of these agents (IgM or acute phase IgG) were also detected: EBV (based on VCA IgM) (n=3), enterovirus (n=6), parvovirus B19 (n=1), *C burnetii* (based on phase II IgG) (n=12). Of the 12 patients who were positive for *C burnetii* phase II IgG, five had Q-CFS/ME. There were no acute infections detected in the normal group.

Regarding EBV serology, there were also associations between CFS/ME subtype and both EBV VCA IgM titre (p=0.0038) and EBV EBNA IgG titre (p=0.0011) (figure 2D). Using the EBV markers VCA IgM, VCA IgG, early antigen IgG and EBNA IgG, we determined the EBV serostatus of infection for each subject (ie, seronegative, primary infection/re-activation, late phase of infection). Among 111 of these CFS/ME patients, there were 11 seronegative, 61 primary/re-activation and 39 late phase of infection, as compared with the normal group, in which there was one seronegative, eight primary/re-activation, and 19 late phase of infection (χ^2=9.91, degrees of freedom=2, p=0.007) (figure 2E).

The distribution of CFS/ME patients by EBV serostatus category (seronegative, primary/re-activation and late phase of infection) across the eight CFS/ME genomic subtypes is shown in figure 2E. In the normal controls, the predominant category of EBV serostatus was late phase of infection, whereas in the CFS/ME subtypes, the predominant category of EBV serostatus was primary/re-activation, which was seen in subtypes A, B, C, D, F and H. Subtype G had equal numbers of primary/re-activation and late phase, and subtype E had a predominance of late phase subjects, but also had five seronegative subjects. This distribution was found to be almost statistically significant (χ^2=25.9, degrees of freedom=16, p=0.055).

EBV-associated genes in each CFS/ME subtype

Within the CFS/ME-associated gene signature of 88 human genes, there were 12 that have recognised associations with EBV infection; these associations have been summarised previously.[7]

Table 3 Fold-difference values for 88 genes in each of eight subtypes (A—H) in 114 subtyped patients with chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME). Genes without values for the subtypes are those for which there was missing data for one or more subtypes. Bold type indicates genes targeted by existing drugs and those CFS/ME subtypes in which fold-difference values of 1.5 were found

Gene symbol	Genbank accession	CFS/ME subtype							
		A	B	C	D	E	F	G	H
ABCD4	NM_020323	1.40	0.60	0.81	4.40	10.61	7.02	1.32	0.93
ACTR3	NM_005721	10.17	8.03	4.34	25.73	13.95	6.75	27.57	5.35
AKAP10	NM_007202	3.83	3.25	1.68	8.39	5.59	6.61	4.89	1.64
ANAPC11	NM_016476	2.53	2.66	4.34	3.74	1.59	4.16	2.27	
ANAPC5	NM_016237	1.13	1.26	4.49	1.92	0.78	0.00	2.96	2.95
APP	NM_201413	0.63	**1.73**	1.42	0.56	**4.64**	0.84	0.84	**2.58**
ARL4C	NM_005737	6.62	7.40	2.72	5.85	7.82	8.76	6.68	2.81
ARPC5	NM_005717	2.55	0.81	1.53	5.65	4.87	3.41	4.92	3.01
ARSD	NM_001669	0.64	1.17	1.51	4.60	2.62	0.26	0.12	2.19
ATP6V1C1	NM_001695	1.99	3.80	3.38	2.43	2.06	0.72	3.63	4.55
BCOR	NM_017745	1.42	2.22	1.78	2.30	6.52	1.13	2.65	2.57
BMP2K	NM_198892	8.96	4.83	2.62	16.42	7.76	3.78	11.57	2.06
BRMS1	NM_015399	1.31	4.38	2.44	1.53	4.62	7.06	2.57	3.83
CD2BP2	NM_006110	3.89	1.37	2.21	6.77	4.52	1.33	5.99	3.55
CD47	NM_198793	2.60	6.90	3.37	3.66	4.06	0.95	4.13	2.75
CEP350	NM_014810	3.50	4.47	3.66	5.30	4.50	1.22	5.04	2.04
CITED2	NM_006079	6.43	6.84	1.97	4.95	6.02	4.40	5.50	3.42
CMTM6	NM_017801	3.10	3.70	0.69	10.04	7.81	4.77	1.71	0.73
CREBBP	NM_004380	7.11	1.62	1.09	13.34	5.46	2.61	8.99	3.62
CRK	NM_016823	1.83	5.57	1.26	2.82	4.89	1.03	2.25	2.02
CTBP1	NM_001328	4.95	4.98	1.05	8.62	15.44	3.43	2.42	2.73
CXCR4	NM_003467	**13.47**	**2.18**	**2.03**	**28.10**	**17.57**	1.48	**10.29**	**3.57**
EBI2	NM_004951	5.67	1.41	2.31	14.93	5.99	0.76	0.42	4.47
EGR1	NM_001955	0.49	2.85	2.42	0.30	0.27	1.00	1.98	2.96
EGR3	NM_004421	0.95	1.33	0.46		5.36		1.39	0.98
EIF2B4	NM_172195	1.44	0.52	1.33	6.00	3.48	0.15	1.69	2.08
EIF3S10	NM_003750	1.43	4.42	2.10	1.72	1.48	1.25	2.83	6.16
EIF4G1	NM_198241	1.13	3.47	3.52	0.99	1.39	2.30	4.53	14.27
EIF4G3	NM_003760	2.40	0.79	0.77	5.46	27.42	1.09	3.62	1.55
ETS1	NM_005238	35.12	4.63	4.16	52.65	30.36	17.54	24.17	6.91
FAM126B	NM_173822	2.04	0.63	0.91	10.18	5.51	2.59	1.31	0.71
FNTA	NM_002027	1.39	**5.72**	**2.99**	**3.88**	**5.07**	**2.14**	**7.88**	**4.06**
GABARAPL1	NM_031412	6.08	6.42	3.37	2.93	8.49	2.58	6.23	5.74
GABPA	NM_002031	11.96	8.56	21.93	3.10	5.71	25.83	13.38	55.99
GCN1L1	NM_006836	0.80	1.40	1.91	0.44	5.85	1.13	1.59	2.38
GLTSCR2	NM_015710	3.68	2.46	0.53	4.94	6.98	4.07	10.28	0.80
GNAS	NM_080425	1.72	1.13	1.81	3.62	3.18	1.96	3.47	2.20
GSN	NM_198252	1.73	1.27	1.69	3.82	2.36	1.64	3.51	5.81
GTF2A2	NM_004492	0.71	0.53	1.21	0.53	2.19	0.48	1.65	0.87
HIF1A	NM_001530	2.04	0.87	0.82	5.14	4.22	1.66	4.65	1.35
IFNAR1	NM_000629	1.86	0.17	0.79	3.55	5.53	1.41	7.17	0.91
IL10RA	NM_001558	1.12	2.68	0.74			2.31	1.01	1.76
IL6R	NM_000565	2.19		2.47			2.78	2.67	
IL6ST	NM_002184	**2.61**	1.49	**4.81**	**3.14**	**3.07**	**2.67**	**3.76**	
IL7R	NM_002185	1.43	0.91	1.66			1.46	2.06	1.33
JAK1	NM_002227	9.72	7.84	3.29	28.88	9.80	11.17	18.75	6.19
KHSRP	NM_003685	0.42	1.03	0.91	0.61	1.15	0.75	1.07	1.62
MAPK9	NM_139070	1.16		1.62	1.68	1.83	0.00	2.51	
METTL3	NM_019852	0.81	1.38	0.64	1.72	2.92	1.08	1.81	0.68
MRPL23	NM_021134	2.34	0.97	1.23	4.15	4.20	1.56	2.56	2.06
MRPS6	NM_032476	2.03	0.92	2.93	3.05	7.75	1.77	1.95	2.00
MRRF	NM_138777	10.11	13.30	3.96	2.03	9.28	1.33	9.70	7.34
MSN	NM_002444	3.20	1.66	1.81	9.47	7.86	3.13	8.58	2.12
MTMR6	NM_004685	3.71	11.73	2.61	7.73	7.33	2.67	14.97	2.58
NFKB1	NM_003998	3.74	0.91	0.65	8.83	6.51	4.10	7.30	1.55
NHLH1	NM_005589	26.32	37.92	49.09			66.39	51.25	126.29
NR1D2	NM_005126	1.40		2.31	4.57	3.69	1.27	2.50	2.29
NTE	NM_006702	1.75	0.31	0.89	3.92	4.37	1.30	3.87	1.43
NUFIP2	NM_020772	1.55	1.90	1.81	2.83	2.10	1.84	3.50	2.31

Continued

Table 3 Continued

Gene symbol	Genbank accession	CFS/ME subtype							
		A	B	C	D	E	F	G	H
PAPOLA	NM_032632	0.47	0.52	0.29	0.79	4.73	0.58	1.25	0.32
PDCD2	NM_002598	3.83	3.44	2.94	5.00	5.74	5.88	5.36	7.37
PDCD6	NM_013232	1.96	2.72	2.53	2.16	4.79	2.69	2.85	2.16
PEX16	NM_004813	2.10	16.10	2.04	8.88	5.92	0.00	2.90	2.80
PGM2	NM_018290	3.23	3.62	2.16	5.99	5.72	4.89	6.13	3.36
PIK3R1	NM_181523	2.06	4.55	0.58	7.17	7.31	0.95	5.48	0.82
PKN1	NM_213560	2.25	3.76	1.27	6.67	6.09	2.39	8.14	2.84
POLR2G	NM_002696	1.09	5.58	2.06	1.91	6.01	2.60	3.82	2.91
PPP2R5C	NM_002719	2.78	2.62	1.28	9.50	6.14	1.63	7.87	0.84
PRKAA1	NM_006251	2.14	4.10	3.42	3.53	4.17	6.87	7.13	3.11
PRKAR1A	NM_002734	2.05	1.85	2.56	4.14	3.66	2.41	6.35	5.41
PUM2	NM_015317	2.81	1.69	0.87	2.85	5.04	1.49	3.84	2.22
RAP2C	NM_021183	2.69		2.56	4.75	5.35	25.28	10.61	1.95
REPIN1	NM_013400	2.37	3.85	3.12	1.92	6.62	8.53	7.06	6.52
RNF141	NM_16422	3.64	0.64	2.10	9.85	11.45	6.08	10.83	2.09
SELENBP1	NM_003944	7.88	9.51	3.46	22.18	7.54	2.84	7.65	5.70
SFXN1	NM_022754	1.37	3.46	1.58	1.35	1.40	1.67	1.99	1.72
SHPRH	NM_173082	0.82		1.07	0.21	7.17	0.00	0.64	
SNAP23	NM_003825	3.46	0.45	1.89	12.62	13.33	4.15	10.19	1.43
SORL1	NM_003105	1.40	1.91	1.60			2.01	1.52	2.47
SOS1	NM_005633	0.70		0.81	1.69	1.09	0.29	1.61	0.90
TAF11	NM_005643	0.56		1.35	1.05	2.13	0.00	0.21	1.23
TCF3	NM_003200	2.00	0.94	1.08	2.83	3.96	3.54	2.52	2.65
TDP1	NM_018319	1.60	5.50	1.38	4.80	11.55	0.96	4.24	
TNFRSF1A	NM_001065	**11.96**	**4.07**	1.36	**18.01**	**13.25**	**3.30**	**17.81**	**2.06**
UBTF	NM_014233	2.88	3.59	1.82	6.03	6.46	4.81	10.91	6.44
USP38	NM_032557	2.66		0.71	7.40	4.27	7.18	2.94	1.02
WAPAL	NM_015045	2.97		5.13	3.63	2.78	1.24	6.04	2.97
WDR26	NM_025160	0.84	0.09	0.63	2.18	1.53	0.80	2.74	1.23

The fold-difference values for each of these 12 genes in each CFS/ME subtype/normal were analysed for significant associations using ANOVA. With all 12 genes, there was a trend which did not reach significance (df=89,p=0.119). However, when *GABPA* and *EGR1* were removed from the analysis, the remaining 10 genes showed a striking association with subtype (ANOVA, df=73, p=0.0001) (figure 2F).

DISCUSSION

We have previously reported the differential expression of 88 human genes in CFS/ME and evidence of clinically relevant subtypes.[7 8] In the present study, we have confirmed this differential expression in 62 additional and previously untested CFS/ME patients. Combining the previous cohort and the new cohort, we have found evidence of eight genomic CFS/ME subtypes with marked differences in global functioning, clinical symptoms, levels of severity and geographical distribution. The function of these genes and their networks has been published previously.[7]

We have addressed the question of the specificity of these 88 genes to CFS/ME, by testing drug-free patients with endogenous depression. The fact that only five of these genes were

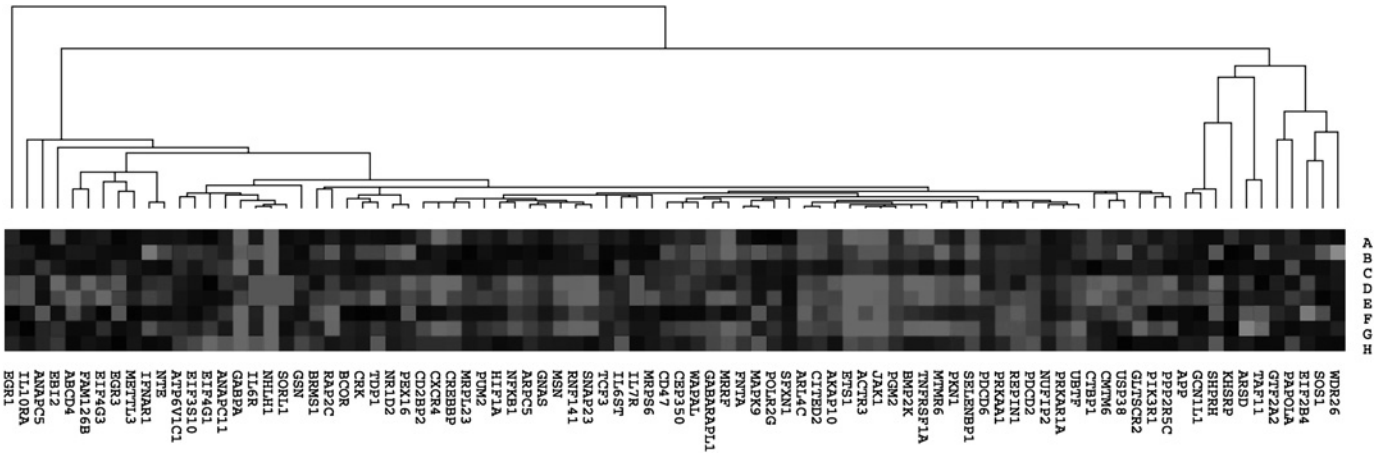

Figure 1 Absolute fold-difference values (mean relative quantity (RQ) in patients with chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME)/ mean RQ in normal controls) for each of 88 CFS/ME-associated genes in eight CFS/ME subtypes (A–H).

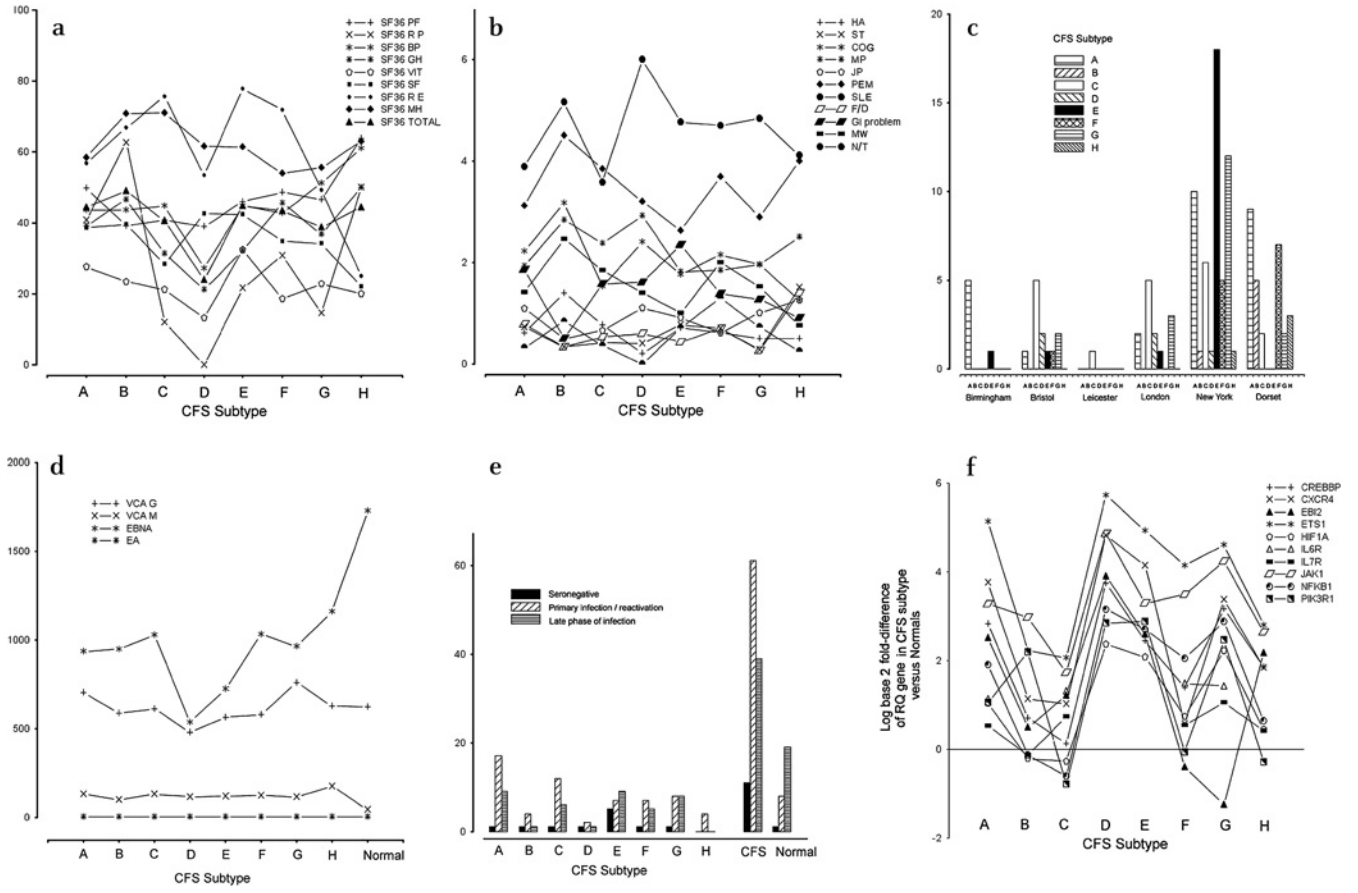

Figure 2 (a) Medical Outcomes Survey Short Form-36 (SF36) domain and total scores for each chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) subtype: physical function, physical role (RP), bodily pain (BP), general health (GH), vitality (VIT), social functioning (SF), emotional role (RE), mental health (MH) and total score (Total). (b) Scores indicating occurrence and severity of 11 clinical symptoms for each CFS/ME subtype: headache (HA), sore throat (ST), swollen glands (GLA), cognitive defect (COG), muscle pain (MP), joint pain (JP), muscle weakness (MW), post-exertional malaise (PEM), sleep problems (SLE), fainting/dizziness (F/D), gastrointestinal complaints (GI), numbness/tingling (N/T), spatial span (SSP), verbal recognition memory (VRM). (c) Histogram showing the numbers of CFS/ME patients of each subtype occurring in each of the six geographical locations. (d) Epstein–Barr virus (EBV) antibody titres (viral capsid antigen (VCA) IgM, VCA IgG, early antigen (EA) IgG, Epstein–Barr nuclear antigen (EBNA) IgG) in each CFS/ME subtype and the normal comparison group. (e) Distribution of categories of EBV serostatus (seronegative, primary/re-activation, late phase of infection) in the CFS/ME subtypes, A–H, in CFS/ME (all subtypes combined) and in normal controls. (f) Log (base 2) of fold-difference values of 10 human genes known to be important in EBV infection, in eight CFS subtypes (A–H).

abnormally expressed in these patients, as compared with normal controls, supports the view that CFS/ME and endogenous depression are biologically distinct, and that the psychological features of CFS/ME are in fact secondary to the pathogenesis.

It is particularly interesting that five of six CFS/ME patients with Q-CFS/ME clustered in the same subtype (subtype A). As these patients had had CFS/ME for several years, this finding suggests that they have a common underlying theme, which may be stable for a long time after the onset of disease. In view of this, and as various genes within this human gene signature are closely linked with EBV infection (*NFKB1*, *EGR1*, *ETS1*, *GABPA*, *CREBBP*, *CXCR4*, *EBI2*, *HIF1A*, *JAK1*, *IL6R*, *IL7R*, *PIK3R1*) and enterovirus infection (*EIF4G1*), we tested the serum samples for markers for four treatable microbial infections that are well recognised to trigger CFS/ME (EBV, enterovirus, parvovirus B19 and *C burnetii* (the agent of Q fever)) with the hypothesis that these genomic CFS/ME subtypes may represent host responses to particular infectious agents.

One patient with subtype E had acute parvovirus B19 at the time of sampling. This patient's symptoms were typical of CFS/ME, but this is not unexpected as parvovirus B19 is a recognised trigger for CFS/ME.[13] The importance of testing for these infections is illustrated here, as we have shown previously that B19-CFS/ME is highly responsive to treatment with intravenous immunoglobulin.[14]

Six patients had acute enterovirus infections (of undetermined serotype) at the time of sampling, but there was no subtype relationship, as two patients were found to have each of subtypes A, E and G. Enteroviruses have long been recognised to trigger CFS/ME,[15] and they have been detected in the stool[16] and stomach epithelium[17] in CFS/ME patients. Detection in the stomach has been shown to be associated with gastrointestinal symptoms in CFS/ME patients.[17] However, in the present study, patients of subtypes A, E and G did not show gastrointestinal symptoms more often than the other patients.

Twelve CFS/ME patients and one normal subject had IgG to *C burnetii* phase II antigen, suggesting possible acute infection. Five of these CFS/ME patients were among those with Q-CFS/ME. The patients in whom these antibodies were detected had subtypes A, B, D, E and G. Therefore, apart from the patients with Q-CFS/ME (whose CFS/ME disease onset was associated with laboratory-documented acute Q fever), there were no subtype-specific relationships with *C burnetii* antibodies.

Take-home messages

- Expression of 88 human genes was confirmed as being significantly different between patients with chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) and normal controls.
- Gene expression in patients with endogenous depression was similar to that in normal controls.
- CFS/ME patients can be grouped into genomic subtypes which have different clinical phenotypes.
- There was evidence of subtype-specific relationships for Epstein–Barr virus and enterovirus, the two most common triggers for CFS/ME.

The subtype associations with EBV and EBV-linked genes are interesting, suggesting differences in the role of EBV and consequent host responses in the different subtypes. The finding of a noticeably large proportion of CFS/ME patients who were EBV seronegative (10%), compared with 4% in the normal group, was quite surprising given the strong link between EBV and CFS/ME. The fact that five of these 11 seronegative cases were subtype E is interesting, but remains unexplained at present.

It has been recognised for some time that subtypes of CFS/ME exist, and it has been thought that these subtypes may, at least in part, reflect particular aetiological factors.[18] A symptom-based approach has had some success in identifying musculoskeletal, inflammatory and neurological subtypes[19]; however, these groups had only minor differences in overall functional severity in contrast with those of the present study.

It is intriguing that it is possible to identify CFS/ME subtypes on the basis of expression values for these 88 genes, and even more so that these subtypes have distinct clinical phenotypes, with marked differences in the occurrence of particular symptoms and their severity. However, what precise sequence of events is involved in the genesis of the gene signatures in each subtype remains to be elucidated. Further work is required to validate and develop these findings.

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Competing interests None.

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Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

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