

Analysis of Val158Met genotype polymorphisms in the *COMT* locus and correlation with IL-6 and IL-10 expression in fibromyalgia syndrome

Ferran J Garcia-Fructuoso¹, Katrin Beyer², Jose I Lao-Villadóniga³

Summary

We have determined Val158Met *COMT* polymorphism genotypes (HH or Val/Val, HL or Val/Met, LL or Met/Met) in 46 fibromyalgia syndrome (FS) patients and 40 controls. A correlation between these alleles and Interleukin-6 (IL-6) and Interleukin-10 (IL-10) expression levels, determined by real-time RT-PCR, has been established. The accumulation of Met-allele carrying genotypes (both heterozygous and

homozygous) observed in FS patients revealed an almost 4-fold increase of risk of developing FS. The Met(-) group of FM patients presented significantly increased IL6 expression levels when compared to all other subgroups. Significantly lower IL-10 expression levels were observed in FS patients, especially in those FS cases carrying the Met allele in comparison with FS Met(-) patients and both control groups.

Keywords: *COMT* polymorphism, Met allele, IL-6, IL-10, fibromyalgia

Accepted for publication: 31 January 2006

¹ Rheumatology Department, CIMA Clinic, Barcelona, Spain

² Hospital Universitari Germans Trias i Pujol, Autonomous University of Barcelona Department of Pathology, Spain

³ Department of Molecular Genetics and Hereditary Pathologies, Laboratorio de Análisis Clínico Dr Echevarne, Barcelona, Spain

Address for correspondence: FJ Garcia-Fructuoso, Fibromyalgia and Chronic Fatigue Syndrome Unit, Rheumatology Department, Clínica CIMA, Paseo Manuel Girona, 33 08034-Barcelona, Spain. E-mail: ferran.garcia@cimaclinic.com

Introduction

Fibromyalgia syndrome (FS) is a chronic, painful, musculoskeletal disorder characterised by widespread pain, pressure, hyperalgesia, allodynia, morning stiffness and an increased incidence of dysthymia symptoms. However, the aetiology has remained elusive and treatment remains mainly empirical¹. FS affects a significant percentage of the general population. It is characterised by chronic diffuse pain and, on examination, areas of focal tenderness called tender points that can be demonstrated in characteristic locations. It occurs predominantly (80–90%) in women of childbearing age (between the ages of 40 years and 60 years)^{2,3}.

There are many theories regarding the aetiology of FS. Some scientists believe that a gene or genes might be involved in fibromyalgia⁴. These genes could make a person react strongly to stimuli that other people would not find painful⁵. According to this observation, some researchers have focused their attention on dysregulation of the autonomic (sympathetic) nervous system as the cause of FS⁶. Fibromyalgia has neuropathic pain features since it is a stimulus-independent pain state accompanied by hypersensitivity to palpation or thermal stimuli (allodynia), and abnormal sensations such as tingling, burning or electric shocks. There are important similarities between fibromyalgia and the localised painful syndrome named Complex Regional Pain Syndrome Type I⁷.

The level of catechol-O-methyltransferase (COMT) enzyme activity is genetically

polymorphic in human tissues, with a trimodal distribution of low (COMTLL), intermediate (COMTLH) and high (COMTHH) activities. This polymorphism, which according to segregation analysis of family studies is caused by autosomal co-dominant alleles, leads to three- to four-fold differences in COMT activity in human erythrocytes and liver. Low COMT activity is associated with enzyme thermolability, even at 37 °C.

Polymorphism in COMT activity could have clinical implications, although the true relationships in approximately 30 genetic mapping studies for a number of diseases have not been very impressive. There are, however, some disorders in which some relationship has been observed (breast cancer susceptibility⁸, Parkinson's disease⁹ and migraine¹⁰).

It has been postulated that people with a COMTLL (Met/Met) or COMTLH (Met/Val) genotype were less able to tolerate pain than persons with a COMTHH (Val/Val) genotype⁵. Positron emission tomography scans showed that this occurred because people with COMTLL (Met/Met) or COMTLH (Met/Val) made less beta-endorphin¹¹. In addition, the decreased COMT levels allowed noradrenaline produced by the sympathetic nervous system to last much longer in their bodies, since noradrenaline is normally destroyed by COMT.

Pro-inflammatory cytokines, such as interleukin (IL)-6, may induce hyperalgesia, for example by acting on the forebrain tissue surrounding the lateral and third

ventricle, and may directly influence the responsiveness of nociceptive neurons^{12–15}. Although cytokines are suspected to play a role in FS, their precise dynamics has escaped elucidation^{16–18}. In experimental animals or humans, IL-6 may induce not only hyperalgesia but also other symptoms characteristic of FS, such as fatigue, sleep disorders and depression-like symptoms¹⁹. IL-10 is a powerful anti-inflammatory cytokine²⁰. As IL-10 is known to suppress the production and release of all three pro-inflammatory cytokines²⁰, it may be predicted to be efficacious for the treatment of neuropathic pain²¹.

It is now well established that the central nervous, endocrine and immune systems interact with each other; psychological stress can downregulate the immune response by affecting the interplay of these systems. The interactions are complex, involving both the hypothalamic–pituitary–adrenal axis and the autonomic nervous system²². Cytokine interactions with the body's pain, hormonal and stress response mechanisms are complex. Sensitised spinal cord neurones in the dorsal horn are responsible for augmented pain processing of nociceptive signals from the periphery. In addition, glial activation, possibly by cytokines and excitatory amino acids, may play a role in the initiation and perpetuation of this sensitised state²³.

A better understanding of these important neuroimmune interactions may provide relevant insights into future effective therapies for FS. For that reason, in the present work we have determined genotypes at the *COMT* locus in a sample of

46 FS patients and 40 healthy controls and correlated these with IL-6 and IL-10 expression.

Methods

Patients

The study included 46 patients (age range, 40–60 years; male:female ratio, 1:8) with clinical diagnosis of FS according to American College of Rheumatology criteria¹ as well as 40 control subjects (age range, 40–60 years; male:female ratio, 1:8) with no FS or chronic fatigue syndrome disorder.

Written informed consent was obtained from all patients and controls included in the present study.

Genotyping

Genomic DNA was extracted from peripheral blood cells according to standard procedures. The following primers were used for *COMT* genotyping: *COMT-U* (forward primer): 5'-GCC CGC CTG CTG TCA CC-3'; and *COMT-L* (reverse primer): 5'-CTG AGG GGC CTG GTG ATA GTG-3'. The polymerase chain reaction (PCR) reaction mixture contained 10x enzyme buffer and primers as well as 5 mM MgCl₂ and 1 U of *EcoTaq Taq* DNA polymerase. PCR conditions were a denaturation step of 5 min at 94 °C followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s. The obtained 238 bp fragment was digested with *Nla*III at 37 °C for at least 3 h. Restriction fragments were visualised on 3% agarose gels.

RNA isolation

TRI Reagent (Molecular Research Center

(MRC), Cincinnati, OH) was used to facilitate isolation of RNA. Blood cells were obtained from 5 ml of whole blood and mixed with 1.0 ml of TRI Reagent according to the manufacturer's protocol. Briefly, mixtures were incubated for 5 min at room temperature and then centrifuged at 12,000 \times g for 10 min at 4 °C to pellet insoluble material and high molecular weight DNA. Phases were separated by centrifugation after addition of 1-bromo-3-chloropropane (MRC). The organic and interphases were discarded and RNA was precipitated with isopropanol and re-suspended in an appropriate volume of diethylpyrocarbonate-treated water. RNA quantity was determined spectrophotometrically at A_{260} ; RNA purity was ascertained from the optical density ratio at 260 nm and 280 nm; and RNA integrity was checked by electrophoresis on an agarose gel. The samples were stored at -80 °C until use.

Real-time reverse transcription PCR

First-strand cDNA synthesis was carried out using Ready-to-go™ You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Two micrograms of RNA was incubated with random hexamers and the First-Strand Beads at 37 °C for 1 h. The resulting cDNA was either used immediately for PCR or stored at -20 °C until use.

The following primers were designed for specific detection of IL-6 and IL-10: IL6-U (forward) TCA ATG AGG AGA CTT GCC TG; IL6-L (reverse) GAT GAG TTG TCA TGT CCT GC; IL10-U (forward) AGC TGA

GAA CCA AGA CCC AGA; and IL10-L (reverse) GGG CTG GGT CAG CTA TCC. Primer sequences were checked against the GenBank database to ensure lack of cross-reactivity with other known human sequences. β -Actin was used as the reference gene and competitor technology was used for adjustment of relative expression data^{24,25}. Real-time PCR reactions were carried out on a LightCycler System (Roche Applied Science, Mannheim, Germany) with the aid of a LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science). Reaction mixtures had a final volume of 10 μ l and contained 3 mM MgCl₂, 10 pmol of each primer and 0.5 μ l of cDNA obtained during reverse transcription. A standard LightCycler programme set of 29 cycles was used, with 50 °C as the amplification programme annealing temperature and 98 °C as the melting curve target temperature (the melting temperature of the β -actin fragment used was as high as 92 °C). All assays were performed twice to ensure their reproducibility and to minimise possible errors, and a negative control was included in each run.

Statistical analysis

Melting peak areas were obtained on the LightCycler instrument using polynomial analysis with background corrections. Relative levels of expression for IL-6 and IL-10 were obtained as ratios between the respective RNA melting peak area and the β -actin competitor melting peak area. All data are shown as mean \pm standard error of the mean values. Analysis of variance (ANOVA) was used to evaluate differences among means. If ANOVA showed

significant differences, pairwise comparisons between means were tested by Tukey B post-hoc test. The null hypothesis was rejected at the 0.05 level of significance.

To analyse the association between *COMT* genotypes in FS and control groups, genotype frequencies were determined by genotype counting. Analyses were conducted by comparing the Val/Val and Met/Met homozygous genotypes and the Val/Met heterozygous genotype.

Logistic regression was used to assess the main interactive effects of *COMT* genotypes and the main interactive effects of IL-6 and IL-10 levels with these genotypes. The χ^2 test was utilised to evaluate Hardy–Weinberg equilibrium and to assess further any interactions shown by logistic regression

analysis. Analyses were performed using SPSS for Windows release 12.0.

Results

COMT genotyping

As expected, differences between *COMT* allele frequencies for cases and controls, with Met allele accumulation in FS patients, were observed. Although only a small number of individuals have been included in the present study, our values corroborated the influence of the *COMT* (Met) allele as a FS risk factor (Table 1). Furthermore, as shown in Table 2, accumulation of *COMT* allele carrying genotypes (both heterozygous and homozygous) observed in FS patients revealed an almost four-fold increased risk of developing FS.

Table 1. Catechol-O-methyltransferase (*COMT*) allele frequencies in fibromyalgia syndrome patients and controls

	<i>n</i>	Allele frequency		<i>p</i> -value ^a	OR ^b	95% CI
		Val (H)	Met (L)			
Patients	46	0.52	0.48			
Controls	40	0.65	0.35	0.003	2.2	1.19–5.78

95% CI, 95% confidence interval.

^a Value for χ^2 test of differences in Met allele frequencies between cases and controls.

^b Odds ratio estimate for the effect of the Met allele frequency on risk for fibromyalgia syndrome.

Table 2. Catechol-O-methyltransferase (*COMT*) genotype frequencies in fibromyalgia syndrome patients and controls

	<i>n</i>	Genotype frequency		<i>p</i> -value ^c	OR ^d	95% CI
		Met(+) ^a	Met(-) ^b			
Patients	46	0.74	0.26			
Controls	40	0.55	0.45	<0.001	3.83	1.90–7.03

95% CI, 95% confidence interval.

^a Met allele carrying genotype (Met/Met and Met/Val).

^b Non-Met allele carrying genotype (Val/Val).

^c Value for χ^2 test of differences in Met allele carrying genotype frequencies between cases and controls.

^d Odds ratio estimate for the effect of the Met allele carrying genotype frequency on risk for fibromyalgia syndrome.

IL-6 expression

A slight but non-significant correlation was found between FS and increased IL-6 expression compared with controls (2.96 ± 0.36 in FS patients vs. 2.47 ± 0.28 in controls, $p = 0.09$; Figure 1). Further analysis consisted of dividing both the FS patients and the controls depending on the presence of the L (Met) allele: 34 FS patients were Met(+) (L allele carriers) and 12 were Met(-); 22 control subjects were Met(+) and

18 were Met(-). Figure 2 shows the results of this genotype-dependent analysis, which revealed that the Met(-) group of FS patients presented significantly increased IL-6 expression levels compared with all other subgroups (3.52 ± 0.18 in FS Met(-) patients vs. 2.68 ± 0.23 in FS Met(+) patients, 2.51 ± 0.34 in Met(+) controls and 2.43 ± 0.30 in Met(-) controls, $p = 0.04$, $p = 0.02$ and $p = 0.009$, respectively).

Figure 1. Relative interleukin (IL)-6 levels in fibromyalgia syndrome (FS) patients and control subjects.

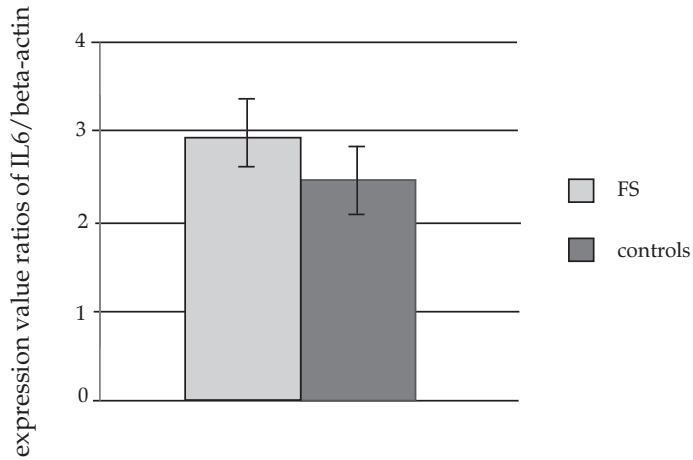
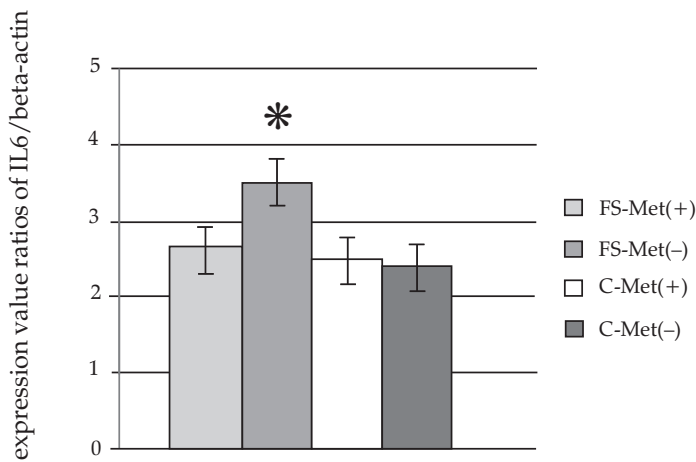


Figure 2. Relative interleukin (IL)-6 levels in fibromyalgia syndrome (FS) patients and control (C) subjects by catechol-O-methyltransferase (COMT) genotype (* $p < 0.05$).



IL-10 expression

No significant differences between IL-10 expression levels for FS patients and controls were observed (0.96 ± 0.12 in FS patients vs. 1.25 ± 0.16 in controls, $p = 0.21$; Figure 3). However, division into *COMT* genotype-dependent subgroups revealed

the influence on IL-10 levels (Figure 4). Significantly lower IL-10 levels were observed in FS patients, the L (Met) allele without compared with FS Met(+) patients and both control sub-groups (0.78 ± 0.11 in FS Met(-) patients vs. 1.17 ± 0.15 in FS Met(+) patients, 1.09 ± 0.09 in Met(+) controls vs. 1.28 ± 0.11 in Met(-) controls).

Figure 3. Relative interleukin (IL)-10 levels in fibromyalgia syndrome (FS) patients and control subjects.

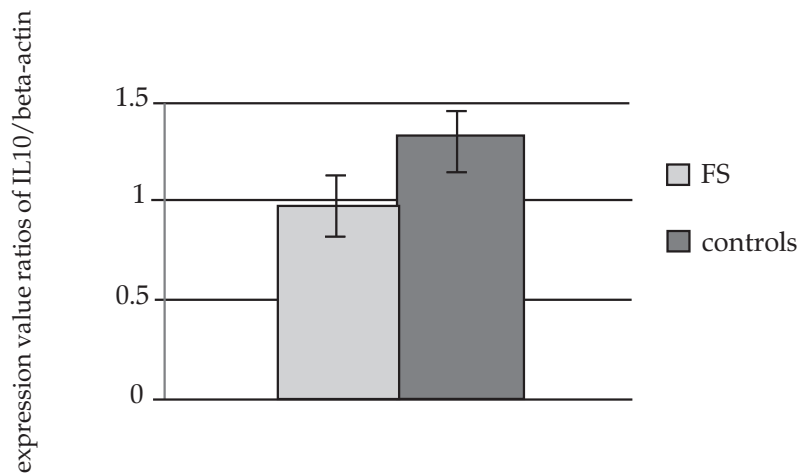
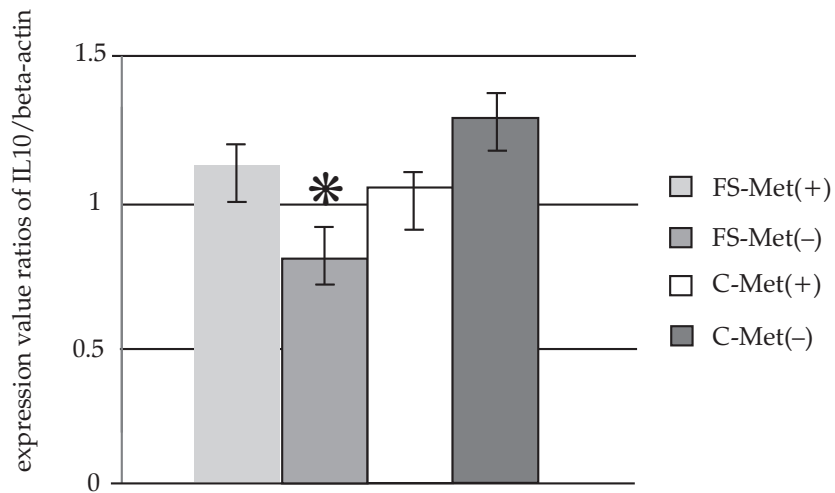


Figure 4. Relative interleukin (IL)-10 levels in fibromyalgia syndrome (FS) patients and control (C) subjects by catechol-O-methyltransferase (*COMT*) genotype (* $p < 0.05$).



controls and 1.28 ± 0.13 in Met(-) controls, $p = 0.007$, $p = 0.02$ and $p = 0.004$, respectively; Figure 4). No statistically significant differences were observed between control group subjects with and without the COMTL (Met) allele, $p = 0.24$; Figure 4).

Discussion

In this study, we corroborate the previously documented observation of the involvement of COMTLL and COMTLH polymorphism in FS²⁶, since it is responsible for the lowest metabolic activity of the COMT enzyme. In this sense, the COMTL (Met) allele could be considered as a genetic risk factor for some FS cases. Nevertheless, this factor may act independently of other factors also involved in the molecular aetiology of FS in different patients.

An increasing number of genetic association studies have implicated polymorphisms in cytokine genes as host genetic factors influencing susceptibility to autoimmunity in many other disorders²⁷. Some researchers have documented that high levels of IL-6 for long periods of time can trigger long-term changes in the body's physiology. These changes lead to age-related disorders such as heart disease, osteoporosis, arthritis, type 2 diabetes, lymphoma, cancer and many others²⁸.

When analysing IL-6 levels, we observed that FS patients without the COMTL (Met) allele presented the highest levels of IL-6, whereas lower IL-6 levels were observed in FS patients carrying the Met (COMTL) allele

as well as in both control subgroups.

IL-10 is a pleiotropic cytokine with well known anti-inflammatory, immunosuppressive and immunostimulatory properties that limits and downregulates inflammation²⁰. High IL-10 levels have been shown to have several protective features against some disorders and some studies have found a link between high IL-10 levels and longevity²⁹. According to the finding in the present study, high levels of IL-10 were observed in healthy controls. In the FS group, patients with the Met allele showed higher IL-10 levels than FS patients with the Val (COMTH) allele and for that reason we supposed that cytokines in particular and immune system disabilities in general do not play a significant role in the aetiopathogenesis of FS in the group of Met(+) patients. In contrast, the immune system appears to be important in the aetiology of FS in patients without the Met (COMTL) allele since they show they highest IL-6 and the lowest IL-10 levels.

These data must be corroborated in a larger sample. Nevertheless, according to these preliminary results, we speculate that phenotypic heterogeneity observed in FS patients might be the result of multiple genetic factors as well as interactions among them. FS appears to be a disorder in which genetic heterogeneity may play an important role in the molecular aetiopathogenesis.

Finally, to test this hypothesis, a significant number of single nucleotide polymorphisms (SNPs) in genes possibly

associated with the molecular aetiopathogenesis of FS must be analysed. Since more than one functional polymorphisms can be expected, it would be of special interest to study SNPs corresponding to aetiopathogenic targets possibly involved in the origin of FS.

Conclusions

The *COMTLL* (Met/Met) and *COMTLH* (Met/Val) genotypes are overrepresented in FS patients.

Expression of IL-6 and IL-10 is different from that expected and it appears to be possible that we are confronted with an illness with multiple aetiologies, multiple origins and various clinical manifestations in which genetic heterogeneity might play an important role. Nevertheless, a more extensive analysis including several SNPs will be necessary to understand the genetic determinants and molecular mechanisms of FS in order to corroborate this hypothesis.

Acknowledgements

This research was supported in part by research funding from the Spanish Fundació per a la Fibromiàlgia i la Síndrome de Fatiga Crònica (<http://www.fundacionfatiga.org>). The authors thank the Spanish Fibromyalgia Association for their kind collaboration.

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