

## Reduced levels of oestrogen receptor $\beta$ mRNA in Swedish patients with chronic fatigue syndrome

Running title: Reduced ER $\beta$  mRNA levels in CFS

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## **ABSTRACT**

### **Background**

Chronic fatigue syndrome (CFS) is an illness with unknown aetiology and pathophysiology. The sex difference observed for CFS indicates a role for oestrogen and oestrogen receptors (ERs) for disease development. Furthermore, an immuno-mediated pathogenesis has been suggested for CFS which provides an additional connection to oestrogen, which displays immunomodular functions.

### **Aims**

The aim of this study was to investigate a possible association of ER mRNAs and two ER $\beta$  single nucleotide polymorphisms (SNPs) with CFS.

### **Methods**

Messenger RNA levels of ER $\alpha$ , ER $\beta$  wt and ER $\beta$  cx were investigated in peripheral blood mononuclear cells (PBMCs) from 30 CFS patients and 36 healthy controls by quantitative real-time PCR. Two ER $\beta$  SNPs were scored in the same material.

### **Results**

The CFS patient group showed significantly lower mRNA expression levels of ER $\beta$  wt compared with the healthy control group. No differences were observed for ER $\alpha$  or ER $\beta$  cx between patients and controls. There were no significant differences in frequency for the investigated ER $\beta$  SNPs between cases and controls.

### **Conclusions**

The reduced ER $\beta$  wt expression levels observed in this study is consistent with an immune-mediated pathogenesis of CFS. Additionally, the observation that ER $\beta$  wt expression is decreased in CFS could provide an entry point to identify interesting, potentially disease causing, candidate molecules for further study. A possible connection between oestrogen, ERs and CFS should be further evaluated.

## INTRODUCTION

Fatigue is a central component of many diseases and illnesses. Fatigue of unknown aetiology and pathophysiology lasting more than six months, together with at least four out of eight specified symptoms, is termed chronic fatigue syndrome (CFS)<sup>1</sup>. The symptoms are impairment of cognition and memory, recurring sore throat, tender lymph nodes, mild muscle pain, joint ache, headaches of a new type, unrefreshing sleep, and post-exertional malaise<sup>1</sup>. Prevalence rates of 0.2-0.5% have been reported in the Western world with a predominance of women (2-4 times higher rates compared with men)<sup>2,3</sup>. The actual target tissues for CFS remain elusive. However, peripheral blood mononuclear cells (PBMCs) have been shown to act as indicators for abnormal biological processes occurring throughout the body<sup>4,5</sup>.

Oestrogen is a steroid hormone that plays important roles in various physiological processes including sexual development and in the reproductive cycle<sup>6</sup>. Oestrogens have been shown to be potential immunomodulators. Several autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS) have an excess of affected females<sup>7</sup>. Furthermore, both MS and RA generally improve during pregnancy, suggesting that oestrogen could play a immunosuppressive role in these contexts (reviewed in<sup>7</sup>). Oestradiol and cyclic progestin treatment has improved health status of premenopausal CFS patients, and one study showed improved health during pregnancy<sup>8</sup>, reviewed in<sup>9</sup>, when oestrogen levels are naturally high. Both CFS and oestrogen have been linked to a Th2 type response of the immune system<sup>7,10,11</sup>.

Oestrogen exerts its effects by binding to the oestrogen receptors (ERs)<sup>6,12</sup>. ERs have been implicated in several diseases presenting unequal proportion of men and women such as breast cancer and osteoporosis<sup>13</sup>. The ERs belong to the nuclear receptor superfamily<sup>12</sup>. There are two ERs,  $\alpha$  and  $\beta$ , which have unique and overlapping roles. For ER $\beta$  there exists a human splice variant, ER $\beta$  cx, which differs at the C-terminal end of the protein<sup>14</sup>. It is unclear what regulates ER $\beta$  wt/ER $\beta$  cx ratios. Possibilities include differential promoter usage and differential mRNA stability. Human ER $\beta$  is expressed from two alternative, tissue specific, first exons, 0N and 0K<sup>15,16</sup>. In this context, Hirata *et al.* have shown that 0N is coupled to ER $\beta$  wt and 0K to ER $\beta$  cx in testis<sup>15</sup>.

Frequency differences between patient and control groups in naturally occurring base-pair changes, referred to as single nucleotide polymorphisms (SNPs), indicate a linkage of the particular genomic region and the disease under study. Association of SNPs in ER $\beta$  with disease has been reported in e.g. anorexic and bulimic patients<sup>17,18</sup>. These diseases also have a female predominance. ER $\beta$  SNPs have also been studied in relation to prostate cancer<sup>19</sup>, Alzheimers disease<sup>20</sup>, pre-eclampsia<sup>21</sup>, efficacy of hormone replacement therapy (HRT)<sup>22</sup>, hypertension<sup>23</sup>, Parkinsons disease<sup>24,25</sup>, breast cancer<sup>26</sup> and as well as other conditions (reviewed in<sup>13</sup>).

Based on the unequal sex distribution for CFS and the reported improvement in health status upon oestrogen treatment, we hypothesized that differential expression of ERs could occur in CFS. In this study we investigate this hypothesis by exploring possible associations between ER mRNA expression levels and/or genetic variants and CFS.

## METHODS

### Study population

The study cohort consisted of 30 CFS patients and 36 voluntary controls stating healthy condition (table 1).

**Table 1:** Study cohort information including clinical patient data such as illness duration, illness onset type, and classification according to ICD-10 system.

	CFS patients* (n = 30)	Controls (n = 36)
Age (years) <sup>†</sup>	40 (26-54)	44 (26-65)
Male/Female	9/21	10/26
Premenopausal (<52 years) / Postmenopausal (>52 years) <sup>‡</sup>	18/3	19/7
Illness duration (years) <sup>§¶</sup>	4.5 (1.5-25)	-
Illness onset type <sup>§**</sup>		
Gradual	15 (4/11)	-
Sudden	9 (2/7)	-
CFS classification (ICD-10) <sup>§**</sup>		
Non-infectious	11 (2/9)	-
Infectious	13 (4/9)	-

\* CFS patients were diagnosed according to the 1994 case definition.<sup>1</sup>

<sup>†</sup> Mean (range)

<sup>‡</sup> 52 years = the mean age for menopause in Sweden

<sup>§</sup> Clinical data were available for 24 patients.

<sup>¶</sup> Median (range)

\*\* Number (male/female)

### Sample preparation

PBMCs were isolated from all patients and healthy controls immediately following blood draw (Venglect® Evacuated blood collection tubes, heparin, Terumo®, Leuven, Belgium) after written informed consent (ethical approval, 130/02, Karolinska University Hospital, Huddinge, Sweden). Ten million PBMCs were lysed (TRIzol® Reagent, Invitrogen, Carlsbad, CA, US) and stored at -80°C. Total RNA was extracted by phase separation and quantified with spectrophotometry (NanoDrop Technologies, Wilmington, DE, US). Twelve samples, one from each extraction batch, were also analysed using the 2100 Bioanalyzer instrument (Agilent Technologies, Palo Alto, CA, US). Complementary DNA was synthesised from total RNA using random primers and the SuperScript™ III system including a DNase treatment step (Invitrogen). Two separate cDNA synthesis reactions were performed on each RNA sample.

### Quantification of mRNA expression

Messenger RNA expression levels were quantified using the ABI real-time PCR system (7700, Applied Biosystems, Foster City, CA, US). TaqMan assays were used for all transcripts. Primers and probes were either designed using the Primer Express® software version 2.0 (Applied Biosystems) (table 2) or purchased from Applied Biosystems (Assays-on-Demand™ Hs01100359 (ERβ wt), Hs01105520 (ERβ cx), Hs99999901\_s1 (18S) and

4310884E (GAPDH)). Samples were run in triplicate. Comparative analysis, according to the ABI Prism 7700 Sequence Detection System (Applied Biosystems), was used to calculate mRNA levels, and the two-sided t-test with unequal variance was used for statistical analysis. Data calculations and statistics were performed in Excel. Serial dilutions of pSG5 hER-plasmid (ER $\alpha$ , ER $\beta$  wt and ER $\beta$  cx) of known concentration were run for absolute quantification of ER mRNA expression levels.

**Table 2:** Primers and probes used for real-time qPCR assays. The probes are dual-labeled oligonucleotides with 5'-FAM and 3'-TAMRA.

<b>Transcript</b>	<b>Forward 5'-3'</b>	<b>Reverse 5'-3'</b>	<b>Probe 5'-3'</b>
ER $\alpha$	GCTAGGAAGTGGGAATGATGAAAG	TCTGGCGCTTGTGTTCAAC	TGGGATACGAAAAGACCGAAGAGGAGGG
ER $\beta$ 0K	GCTCAGGTTACAGTCATCCAAT	CAAGAAGAGGCACAAAGGTCATT	TGGTCTGAAGCCATTATACTTGCCCACG
ER $\beta$ 0N	AAGCACGTGTCCGCATTTTAG	TCTCAAAGATTCGTGGCAAGT	AGGCCGGTGTGTTTATCTGCAAGCCATTAT

### **ER $\beta$ SNP analysis**

Synthesised PBMC cDNA samples were used for analysis of ER $\beta$  SNPs rs4986938 ( $\beta$  wt) and rs928554 ( $\beta$  cx). The SNP analysis was performed as described elsewhere<sup>18</sup>.

## RESULTS

### **ER $\beta$ wt mRNA levels are lower in CFS patients compared with controls**

The CFS patient group showed significantly lower mRNA expression levels for ER $\beta$  wt compared with the healthy control group (figure 1), using either of the control genes for normalisation. This was also true when subdividing the patient and control groups according to sex ( $p_{\text{female}} < 0.007$  and  $p_{\text{male}} < 0.02$ ). The results were repeated with a subsequent cDNA synthesis starting with the same RNA samples. ER $\alpha$  and ER $\beta$  cx mRNA levels did not differ between CFS patients and controls (figure 1). There were no differences in ER mRNA levels between sexes and no correlation with age (data not shown).

ER mRNA levels were also compared between CFS subgroups (table 1). Reduced ER $\beta$  cx mRNA expression levels were observed in the subgroup of patients with shorter illness duration ( $\leq 2.5$  years) compared with the group with longer duration ( $\geq 9$  years). Overall, the ER $\alpha$  and ER $\beta$  cx mRNA expression levels in PBMCs were about 100-fold higher (femtograms) compared with the ER $\beta$  wt mRNA level.

Of the two known promoters for ER $\beta$  only expression from the 0N promoter was detected in PBMCs (data not shown).

### **ER $\beta$ SNPs are not associated with CFS in this cohort**

Since our cohort is relatively small we chose to score only the rs4986938 ( $\beta$  wt) and rs928554 ( $\beta$  cx) SNPs where the allele frequencies for the rare alleles are  $> 35\%$ . There was no significant difference in allele or genotype frequencies between CFS patient and control groups (data not shown). However, it is worth noting that the ER $\beta$  rs4986938 AA genotype was more often found in the CFS group (data not shown).

## DISCUSSION

Significantly reduced levels of ER $\beta$  wt mRNA in PBMCs were found in CFS patients compared with healthy controls (figure 1). Based on this observation we extended our studies to include determination of possible association of ER $\beta$  SNPs and promoter levels with CFS. The levels of ER $\beta$  wt mRNA are low in PBMCs. However, the actual target tissue(s) for CFS are unknown, and it is possible that these tissues express higher amounts of ER $\beta$  wt mRNA, still maintaining the differential expression observed in this study. Lower levels of ER $\beta$  cx mRNA were identified in CFS patients with shorter illness duration compared with patients with longer duration. However, the groups and the difference between the groups are small, so the significance of this finding is at present unclear.

A recent study by Phiel *et al.* shows the presence of ER $\alpha$  and ER $\beta$  mRNA levels in fractionated T- and B-lymphocytes<sup>27</sup>. However, the assay used in the study does not discriminate between ER $\beta$  wt and ER $\beta$  cx. Our PBMC samples contain both T- and B-lymphocytes, and it would be of interest to investigate if there are differences in the ER $\beta$  splice variant ratios between the lymphocyte fractions.

Interestingly, in the present study we find differences in the levels of the ER $\beta$  wt mRNA, but not the splice variant ER $\beta$  cx, when comparing CFS patients and controls. It is unclear how the relative levels of these transcripts are regulated. Our data do not support promoter usage as a means by which ER $\beta$  wt/ER $\beta$  cx ratios are regulated, as we only detect expression from one promoter (ON, data not shown). Consistent with our results, promoter ON has been shown to be used in peripheral leukocytes<sup>15</sup>. Tissue specific promoter usage has, however, been reported and the presence of additional ER $\beta$  promoters must be considered in this context<sup>15,16</sup>. It is possible that SNPs in the differing 3' UTRs of ER $\beta$  wt (rs4986938) and ER $\beta$  cx (rs928554) transcripts regulate mRNA stability and ultimately determine ER $\beta$  wt/ER $\beta$  cx ratios. In this context, the ER $\beta$  rs4986938 AA genotype was over represented in the CFS group with lower ER $\beta$  wt expression. The possibility that this SNP regulates mRNA stability can be furthered explored by directly assaying mRNA stability of mRNAs incorporating the G and A alleles.

Several gene expression studies have been performed in the search for differently transcribed genes between CFS patients and controls<sup>4,28-32</sup>. These studies confirmed the role of the immune system in CFS as several genes involved in immunity and defence were shown to differ between CFS patients and controls.

Oestrogens have been suggested as immunomodulatory factors. This is based on observations including the female predominance of certain autoimmune disorders (reviewed in<sup>7</sup>), which might be explained by lower oestrogen secretion in men. Thus CFS and oestrogen signalling are connected via the unequal sex distribution and their association with aspects of immunomodulation. However, none of the gene expression studies referred to above, identified ER $\beta$  as a differentially expressed gene, which might reflect its low expression level.

At present, specific oestrogen receptor modulators are being developed as novel therapeutics for immune-mediated diseases. In a study by Follettie *et al.*, the specific ER $\beta$  agonist ERB-041 was used to treat antigen-induced arthritis in rats<sup>33</sup>. ERB-041 treatment led to sustained improvement and down-regulation of genes known to be up-regulated in rheumatoid arthritis<sup>33</sup>. ERB-041 also showed positive effects in animal models of inflammatory bowel disease<sup>34</sup>.

In conclusion, the difference in expression of ER $\beta$  wt mRNA between CFS patients and controls observed in this study could contribute to some of the symptoms observed in CFS. It is also possible that reduced levels of ER $\beta$  wt mRNA are simply a marker for changed function of other cellular components, which are involved in CFS. The present finding with lower ER $\beta$  levels displayed in CFS patients compared with controls supports an immunopathogenesis for CFS. However, further work is needed to clarify if reduced ER $\beta$  expression is a primary event in CFS or due to a down-regulation secondary to altered oestrogen levels. The reduced ER $\beta$  wt mRNA levels in CFS patients could provide an entry point to identifying interesting potentially disease causing candidate molecules for further study. Studies to investigate ER $\beta$  wt protein levels and cellular effects will be required to confirm an involvement of ER $\beta$  wt in CFS. Future studies should involve evaluation of oestrogen levels and effects following oestradiol treatment in relation to CFS pathology.

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## **COMPETING INTERESTS**

We declare that we have no competing interests.

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## FIGURE LEGEND

**Figure 1:** Average mRNA expression levels, with bars for the standard error of mean, comparing the entire patient group with the healthy control group for ER $\alpha$ , ER $\beta$  wt and ER $\beta$  cx using 18S rRNA for normalisation. Significantly reduced levels were observed for ER $\beta$  wt, while no differences were observed for ER $\alpha$  or ER $\beta$  cx. Similar results were observed using GAPDH for normalisation (results not shown).

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Figure 1. Gräns *et al*

