Fatigued breast cancer survivors and gene polymorphisms in the inflammatory pathway

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A B S T R A C T

Chronic fatigue (CF) in breast cancer survivors (BCSs) has been associated with increased serum C-reactive protein levels (CRP), pro-inflammatory cytokines and cytokine gene single nucleotide polymorphisms (SNPs). Still, there are few studies on these topics, and due to small study-cohorts the possibility to adjust for other conditions related to inflammatory processes, e.g. depression, has been limited. In 302 BCSs, examined approximately four years after treatment for breast cancer stage II/III, data on high sensitivity (hs)CRP, leukocytes and mRNA interleukin (IL)-1β and IL6 expression, depression and chronic fatigue were available. Three years thereafter, 236 BCSs were re-examined. The associations between fatigue and SNPs in inflammation-related genes; IL1β (rs16944), IL6 (rs1800795), IL6 receptor (rs4129267, rs4845617, rs2228145), CRP (rs2794521, rs3091244) were investigated, together with the relations between SNPs in IL6R, IL1β and CRP genes and mRNA blood expression levels of IL6R and IL1β and serum hsCRP levels, respectively. All analyses were repeated after exclusion of depressed individuals and separating BCSs with persistent fatigue from never-fatigued individuals. Even after exclusion of depressed individuals neither the SNPs nor the mRNA IL1β and IL6 expression levels were associated with chronic or persistent fatigue. In the subset of persistent fatigued and never-fatigued individuals the CRP SNP (rs3091244) was associated with hsCRP level (p = 0.02). IL1β and IL6R mRNA expression levels were not related to the IL1β and IL6R genotypes.

In a large cohort of BCSs the investigated SNPs in inflammation-related genes were not associated with fatigue, though subset analyses indicated an association between the CRP SNP (rs3091244) and serum hsCRP.

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1. Introduction

Fatigue is probably the most prevalent late effect among breast cancer survivors (BCSs). It is associated with impaired functioning and overall quality of life, but relatively poorly understood in terms of etiology and mechanisms (Broeckel et al., 1998; Bardwell and Ancoli-Israel, 2008).

We have recently demonstrated that approximately one-third of extensively treated disease-free BCSs experienced chronic fatigue (CF, defined as increased level of fatigue for ≥6 months (Wessely, 1995)) at 2.5–7 years and at 5–10 years after breast cancer (BC) diagnosis (Reinertsen et al., 2010). Further, 23% of the women reported CF at both assessment points (i.e. persistent fatigue, PF). These findings confirm previous results from other research groups that have studied BCSs with similar observation times (Bower et al., 2000, 2006; Alexander et al., 2009; Servaes et al., 2007). Our BCSs with PF had significantly higher levels of fatigue than BCSs who were not chronic fatigued or had CF at only one assessment, possibly reflecting a more severe condition in these subjects (Reinertsen et al., 2010).

In the same cohort we found that the BCSs with CF had increased high sensitivity C-reactive protein (hsCRP), and that survivors with PF also had increased leukocyte count compared to the non-chronic fatigued (Reinertsen et al., 2010). Further, the chronic fatigued BCSs also had dysregulated B-cell pathways compared to...
the non-chronic fatigued (Landmark-Høyvik et al., 2009). Elevated serum levels of CRP in fatigued BCSs have also been reported by others (Alexander et al., 2009), suggesting that sustained inflammation may be related to fatigue in BCSs.

Cytokines are proteins mainly secreted by activated cells of the immune system, and they may induce fatigue by their effects on the central nervous system (Gabay and Kushner, 1999; Schubert et al., 2007; Levy, 2008). Cytokine dysregulation has been proposed as a possible mechanism for the development of fatigue in BCSs, based on findings of elevated serum levels of interleukin (IL)-1 receptor antagonist, cytokine-related tumor necrosis factor receptor type II and soluble IL6 receptor (sIL6R) in fatigued compared to non-fatigued BCSs (Bower et al., 2002; Collado-Hidalgo et al., 2006). One previous study demonstrated associations between regulatory single nucleotide polymorphisms (SNPs) in the cytokine genes IL1\(\beta\) (rs16944) and IL6 (rs1800795) and fatigue in 47 BCSs (Collado-Hidalgo et al., 2008).

Still, the findings supporting a link between inflammation and fatigue in BCSs are not conclusive due to relatively small sample sizes (N < 50) and partly lack of confirmative findings (Bower et al., 2002; Collado-Hidalgo et al., 2006, 2008). Studies in larger sample-sets have been called for to clarify the associations between fatigue and inflammatory biomarkers (Bower et al., 2011).

In general, inflammation has also been linked to fibrosis (Stramer et al., 2007). Finally, CRP, a marker of inflammation, has been shown to be associated with BMI and with depression together with IL6 (Kathiresan et al., 2006; Howren et al., 2009; Musselman et al., 2001). In our BCSs cohort, we found that discomfort in the BC-treated area (represented by both fibrosis and/or pain) was associated with CF, and PF, and correlated with hsCRP (Reinertsen et al., 2010). Thus, adjustments for fibrosis and BMI seem relevant when exploring the relation between fatigue and inflammation.

A well-defined phenotype definition is essential when exploring genetic mechanisms of fatigue (Landmark-Høyvik et al., 2010; Barsevick et al., 2010). Because fatigue is a subjective perception accompanying several disease processes including acute conditions such as infections, chronic fatigue is theoretically less heterogeneous than an elevated fatigue level. In the current paper, refinement of the fatigue phenotype was endeavored first by performing sub-analyses including only the BCSs with PF and never-fatigued individuals (i.e. those with the highest and lowest fatigue symptom scores). Further, additional optimization of the fatigue phenotype was attempted by excluding possibly depressed BCSs.

The current study aimed to explore the associations between fatigue and SNPs related to the inflammatory pathway in a large cohort of BCSs, among whom significant differences in inflammatory biomarkers previously have been demonstrated between individuals with and without chronic fatigue (Reinertsen et al., 2010). Specifically, we aimed to confirm prior reports of SNPs in cytokine genes (IL1\(\beta\), IL6) being associated with fatigue in BCSs (Collado-Hidalgo et al., 2008). We hypothesized that fatigue in BCSs would be associated with SNPs affecting serum protein levels of IL6R and CRP that previously had been related to fatigue in BCSs (Collado-Hidalgo et al., 2006; Alexander et al., 2009; Reinertsen et al., 2010; Galicia et al., 2004; Ridker et al., 2008; Kathiresan et al., 2006; Hage and Szalai, 2007; Wang et al., 2009). Additionally, we aimed to explore the association between genetic variation in three selected genes and IL1\(\beta\) and IL6R mRNA expression in blood and serum hsCRP levels.

To summarize, we primarily hypothesized that in this cohort of BCSs SNPs in the IL1\(\beta\), IL6, IL6R and CRP genes would be related to fatigue. Secondly, we expected that SNPs in the IL6R and IL1\(\beta\) genes would be associated with mRNA blood expression levels of IL6R and IL1\(\beta\), respectively and that SNPs in the CRP gene would be associated with hsCRP protein serum levels.

2. Material and methods

2.1. Study sample

2.1.1. Part 1 — First assessment (T1)

Women treated for BC stage II/III were in 2004/2005 (T1) invited to attend a follow-up study concentrating on late effects if they fulfilled the following eligibility criteria:

1. Having undergone loco-regional adjuvant radiotherapy at the Norwegian Radium Hospital (NRH) during the years 1998–2002, combined with adjuvant chemotherapy with or without endocrine therapy.
3. No recurrence of BC.
4. No other cancer except for basal cell carcinoma, carcinoma in situ of the uterine cervix, or prior or simultaneous surgery for contralateral BC stage I with no adjuvant treatment.

Details regarding patient selection and treatment have been described previously (Reinertsen et al., 2009). Of the 415 invited women, 317 (76%) eligible subjects completed a mailed questionnaire and attended an outpatient physical examination including whole blood sampling at the NRH. Among these, 15 women were excluded from the current study due to lack of DNA for genotyping, yielding a total of 302 women included in part 1 of this study (Fig. 1).

2.1.2. Part 2 — Second assessment (T2) and refining of the fatigue phenotype

In 2007 (T2), 310 women (registered alive) participating in the 2004/2005 outpatient examination were invited to take part in a second follow-up study by responding to the same questionnaires as at T1. At T2, 236 (76%) eligible women returned the questionnaires for a second time (Fig. 1). To maximize phenotypic differences, this cohort was divided into subjects having chronic fatigue at both assessment points (i.e. persistent fatigue symptoms, N = 55) and those not reporting fatigue at any of the two time points (N = 120), i.e. never-fatigued individuals (Landmark-Høyvik et al., 2009; Reinertsen et al., 2010), thus restricting the sub-analysis to individuals with the highest or lowest fatigue symptom levels.

In both parts of the study analyses were repeated after exclusion of individuals reporting depression at T1 to further refine and optimize the phenotypes.

The Norwegian population is homogenous and similar to North-Europeans with low level of genetic differentiation (Luo et al., 2008). Of the 302 BCSs included in part 1 of the study, only 5 individuals reported non-Norwegian heritage (Greek [N = 1], Iranian [N = 2] and from Great Britain [N = 2]).

2.2. Questionnaires

The BCSs completed the fatigue questionnaire (FQ) which includes seven items assessing physical fatigue and four measuring mental fatigue (Chalder et al., 1993). Each item has four response alternatives ranging from 0 (“less than usual”) to 3 (“much more than usual”), with higher scores implying more fatigue. Total fatigue is the sum score of all eleven items, with 33 as the maximum score.

Chalder et al. (1993) suggested a cut-off point of 4 or higher on a dichotomized scale for case definition (indicative of clinically relevant fatigue), and in line with this the raw FQ scores were dichotomized (0 = 0 or 1, 1 = 2 or 3). The FQ has an additional item assessing the duration of fatigue. Chronic fatigue (CF) was defined
as a sum of dichotomized scores \( \geq 4 \) combined with symptom duration of 6 months or longer (Chalder et al., 1993; Wesely, 1995). A recent review of instruments for assessment of cancer-related fatigue recommended use of the FQ (Minton and Stone, 2009).

Our BCSs with CF at both assessments were defined as having persistent fatigue (PF) (Bower et al., 2006; Reinertsen et al., 2010). The FQ’s Cronbach’s \( \alpha \)’s in the present sample were 0.92 (physical fatigue), 0.87 (mental fatigue) and 0.93 (total fatigue).

Anxiety and depression were measured by the Hospital Anxiety and Depression Scale (HADS) (Zigmond and Snaith, 1983). This questionnaire includes two subscales: anxiety (HADS-A) and depression (HADS-D). Each subscale consists of seven items. Each item has four response alternatives (0 = “not present”, 3 = “severe”), giving sum scores on each subscale from 0 to 21. According to the author’s recommendations, HADS cases were defined as subjects with a sum score \( \geq 8 \) within either scale (Zigmond and Snaith, 1983). Depressed individuals were defined as having a HADS-D score \( \geq 8 \), while HADS-A cases had a HADS-A score \( \geq 8 \). Cronbach’s \( \alpha \)’s in the current study were 0.85 (HADS-A), 0.83 (HADS-D) and 0.89 (HADS total). The HADS has been shown to perform well in assessing caseness of anxiety and depression, and the optimal balance between sensitivity and specificity was confirmed when defining caseness by a score \( \geq 8 \) (Bjelland et al., 2002).

### 2.3. Disease-related data and data from clinical examination

Clinical data were collected from the women’s medical records at the NRH.

At the first assessment, an experienced oncologist scored the presence of tissue fibrosis in the irradiated regions as “none”, “little”, “some” and “substantial”, based upon the LENT SOMA scoring system (Late Effects of Normal Tissues (LENT) Consensus Conference, 1995). The scores for fibrosis were dichotomized (0 = “none/little”, 1 = “some/substantial”). Body mass index (BMI) at T1 was calculated as kg/m^2.

### 2.4. Blood tests

Non-fasting blood samples were drawn at T1. Leukocyte counts were performed with CELL-DYN® 4000 (Abbott diagnostic division, USA). The reference range for leukocyte count was 3.3–11.0 \( \times 10^9 \)/l. Serum levels of C-reactive protein were measured with a high-sensitivity, particle-enhanced immunoturbidimetric assay (hsCRP), (Roche Diagnostica, Basel, Switzerland). The detection limit for hsCRP was 0.2 mg/l.

### 2.5. Genotyping and SNP selection

Peripheral blood was drawn into 10 ml EDTA tubes and DNA was isolated using the Autopure LS DNA Purification System (Gentra Systems, Inc., Minneapolis, MN). The purified DNA was stored at \(-80^\circ\)C until genotyping. Subject samples were identified by sample identification numbers only and the clinical outcome data were not available during the genotyping.

Seven SNPs were genotyped and primers and probes for all selected SNPs are listed in Supplementary Table 1. The CRP rs3091244 SNP was genotyped by Sanger sequencing using Applied Biosystems BigDye® Terminator v1.1 Cycle Sequencing kit (Part No. 4337451) and 3730 DNA Analyzer. The other SNPs were genotyped using TaqMan® SNP Genotyping Assays (Applied Biosystems) on the TaqMan 7900HT Fast Real-Time PCR Platform according to standard genotyping protocol. The PCR reaction was carried out in a 384-well format using 12.2 ng genomic DNA in 5 \( \mu l \) total volume.

Three of the genotyping assays were pre-designed (IL1\( \beta \) rs16944 [A/G], IL6R rs4129267 [C/T] and IL6R rs4845617 [A/G]), while 4 were custom made (IL6R rs2228145 previously denoted rs8192284 [A/C], IL6 rs1800795 [G/C], CRP rs2794521 [C/T] and CRP rs3091244 [A/G/T]). Sequences for the custom assays were found in the SNPper (http://snpper.chip.org/) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) databases.

### 2.6. Expression analyses

In this part of the study we used data generated and made available to us by Landmark-Høyvik et al. (2009). Total RNA from the BCSs had been isolated from PAX tubes at AROS Applied Biotechnology (Aarhus, Denmark) by an automated procedure using the column based technologies from Qiagen Inc. (Hilden, Germany) and further processed as described in detail previously (Landmark-Høyvik et al., 2009). Whole genome mRNA expression
analyses were performed on the Illumina Human-6 version 2 expression bead chips (Landmark-Høyvik et al., 2009). The data included information on mRNA expression levels of \(\text{IL1}_\text{b}\) using a probe annealing to a region in exon 7 (\(\text{IL1}_\text{b}_\text{ex7}\)) and for \(\text{IL6R}\) using probes measuring expression levels in exon 9 and at the 3' UTR region (\(\text{IL6R}_\text{ex9}\) and \(\text{IL6R}_\text{3' UTR}\), respectively) (Supplementary Table 2).

### 2.7. Statistics

Continuous variables are reported as median and range, categorical variables as proportions. Expression data were VST transformed and normalized using the quantile normalization method (Lin et al., 2008). T-tests were applied for testing mRNA expression data in relation to fatigue status. In the remaining analyses Kruskal

### Table 1
Characteristics at T1\(^a\) for the different fatigue groups explored in part 1: chronic fatigue (CF) vs not CF and in part 2: persistent fatigue (PF) vs. never-fatigued.

<table>
<thead>
<tr>
<th>Characteristics at T1</th>
<th>Part 1</th>
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<td>Women without CF</td>
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<td>Women with PF</td>
<td>Never-fatigued women</td>
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<td>25.6 [18.9–49.3]</td>
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<tr>
<td>Some/substantial</td>
<td>13 (13)</td>
<td>18 (9)</td>
<td>0.29</td>
<td>11 (20)</td>
<td>11 (9)</td>
<td>0.045</td>
<td></td>
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<tr>
<td>None/little</td>
<td>88 (87)</td>
<td>183 (91)</td>
<td>0.24</td>
<td>44 (80)</td>
<td>109 (21)</td>
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<tr>
<td>Biochemical findings</td>
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<tr>
<td>hsCRP ((\text{mg/l}))</td>
<td>2.5 [0.2–23.0]</td>
<td>1.6 [0.2–31.0]</td>
<td>0.003</td>
<td>2.7 [0.2–23.0]</td>
<td>1.4 [0.2–20.0]</td>
<td>&lt;0.001</td>
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<tr>
<td>Leukocytes ((10^9/\text{l}))</td>
<td>5.6 [3.0–11.0]</td>
<td>5.3 [2.7–11.5]</td>
<td>0.05</td>
<td>5.8 [3.8–11.0]</td>
<td>5.2 [2.8–10.2]</td>
<td>0.009</td>
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</table>

\(^a\) First assessment point.

\(^b\) Breast cancer.

\(^c\) Hospital Anxiety and Depression scale.

\(^d\) Modified radical mastectomy.

\(^e\) Breast conserving surgery.

\(^f\) Body mass index.
Wallis tests for n-independent samples were used for continuous variables due to few individuals in some of the tested genotype groups. The strength of associations was tested in logistic regression analyses and expressed as odds ratio (OR) with 95% confidence intervals (CI). Chi-square tests were applied for categorical variables. The genotypes were analyzed using an additive model.

All analyses were performed using SPSS 16. The level of significance was set at \( p < 0.05 \), and all tests of statistical significance were two-sided. No corrections for multiple testing were performed.

2.8. Ethics

The Regional Committee for Medical and Research Ethics and the Norwegian Data Inspectorate approved the study. All participants gave written informed consent.

3. Results

3.1. Part 1 – Chronic fatigued vs not fatigued women

Of the 302 women included at T1, 101 (33%) individuals had CF. No differences were found between women with or without CF regarding age at BC, age at survey, follow-up time, cancer- and treatment related variables, or treatment-area-related fibrosis (Table 1). However, compared to the non-fatigued individuals, subjects with CF had higher BMI \( (p = 0.002) \), hsCRP \( (p = 0.003) \) and borderline elevated leukocyte counts \( (p = 0.05) \). In addition, a significantly higher proportion of those with CF were HADS cases compared to those without CF \( (47\% \text{ vs. } 18\%, \ p < 0.001) \).

The genotyping succeeded in 89–94% of the 302 women (Table 2). Mostly, the drop outs were the same individuals, implying low DNA quality in these samples. All SNPs were in Hardy–Weinberg Equilibrium (HWE), except the rs1800795 SNP in the IL6 gene, which showed a minor deviation from HWE \( (HWE = 4.04) \). Minor allele frequencies ranged from 0.04 to 0.48.

There were no differences in genotype distribution for any of the SNPs between women with or without CF \( (p\text{-values 0.46–0.72} \) (Supplementary Fig. 1). Neither were the influence of the tested SNPs on fatigue moderated by the different treatment strategies such as chemotherapy and radiotherapy as assessed by multiple logistic regression analyses.

3.1.1. Genotype vs. mRNA expression of IL1β and IL6R

Gene expression data on IL1β, IL6R_3'UTR and IL6R_ex9 existed in 243 \( (80\%) \) of the 302 women. The IL1β-genotypes were not related to IL1β mRNA expression \( (p = 0.61) \). Neither were the different IL6R-genotypes associated with IL6R mRNA expression levels \( (p\text{-values 0.22–0.95}) \). The CRP rs2794521 SNP was associated with IL1β mRNA expression levels \( (p = 0.04) \), and individuals carrying the minor CC genotype displayed the lowest IL1β mRNA expression levels. No differences were detected between individuals with or without CF regarding mRNA expression level of IL6R or IL1β \( (p\text{-values 0.59–0.91}) \).

3.1.2. Genotype vs. serum hsCRP level

The SNPs in the CRP gene (rs2794521 and rs3091244) were not significantly associated with the hsCRP level \( (p = 0.10 \text{ and } 0.13, \text{ respectively}) \). Neither did SNPs in the other studied genes impact on the hsCRP level \( (p\text{-values 0.26–0.91}) \).

3.1.3. Excluding depressed individuals

By excluding depressed individuals from the 302 participants, 267 subjects remained – 77 women with and 190 women without CF. No differences were found between those with and without CF regarding age at BC, age at survey, follow-up time, cancer- or treatment related variables. Non-depressed women with CF had higher BMI \( (p = 0.006) \) and hsCRP levels \( (p = 0.04) \) and were more often HADS-A cases \( (p = 0.002) \) than those without CF.

Among the non-depressed BCSs, no differences were detected between women with and without CF regarding the analyzed SNPs \( (p\text{-values 0.12–0.65}) \) or regarding the mRNA expression levels of IL1β and of IL6R \( (p\text{-values 0.57–0.70}) \) which were based on data from 215 of the 267 women \( (81\%) \).

Except for the association between CRP rs2794521 and IL1β mRNA expression level \( (p = 0.008) \), none of the other SNPs impacted on IL1β or IL6R mRNA expression levels \( (p\text{-values 0.21–0.93}) \). Neither were any SNPs associated with serum hsCRP level \( (p\text{-values 0.21–0.73}) \).

3.2. Part 2 – Persistent fatigued vs. never-fatigued women

Refining the phenotypic differences within the study sample, by excluding individuals with CF only at one assessment, resulted in a total of 175 remaining women – 55 (31%) subjects with PF and 120 subjects not reporting fatigue at any of the assessment points (i.e. never-fatigued). No differences were found between the subjects with PF and the never-fatigued individuals regarding demographic, cancer- or treatment related variables (Table 1). Women with PF had higher BMI \( (p = 0.001) \), hsCRP \( (p < 0.001) \) and leukocyte levels \( (p = 0.009) \) than the never-fatigued subjects. Also, a higher proportion of women with PF were HADS cases \( (p < 0.001) \) and had more

<table>
<thead>
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<th>Table 2</th>
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<td>Details of the SNPs examined in the total sample of 302 breast cancer survivors.</td>
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<tr>
<td>Gene</td>
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<tr>
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</tr>
<tr>
<td>IL1β</td>
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<tr>
<td>IL6</td>
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<td>IL6R</td>
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<td>IL6R</td>
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<td>IL6R</td>
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<tr>
<td>CRP</td>
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<tr>
<td>CRP</td>
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1 Deviating from HWE.  
2 SNP = single nucleotide polymorphism.  
3 MAF = minor allele frequency.  
4 11 = Homozygosity major allele.  
5 12 = Heterozygosity.  
6 22 = Homozygosity minor allele.  
7 11 = CC, 12 = CT, 22 = TT, 13 = CA, 23 = TA.  
8 HWE = Hardy–Weinberg Equilibrium (95% confidence interval calculated with one degree of freedom for the bi-allelic systems; 3.84, and with three degrees of freedom for the three allele system: 7.82).
severe treatment-area related fibrosis ($p = 0.045$) compared to never-fatigued individuals. Higher levels of hsCRP were significantly associated with PF after adjustment for BMI and treatment-area related fibrosis ($OR = 1.11$, $95\% CI [1.01–1.21]$, $p = 0.03$). The SNPs were not associated with PF ($p$-values 0.69–0.99).

3.2.1. Genotype vs. mRNA expression of IL1$\beta$ and IL6R
mRNA expression data were available for 144 (82%) of the women included in part 2 of the study. One of the SNPs in the IL6R gene (rs4845617) was borderline significantly associated with mRNA expression level of IL6R_3’UTR ($p = 0.05$). The other IL6R genotypes did not impact on the IL6R expression level ($p$-values 0.37–1.00) and the different IL1$\beta$ genotypes were not related to the IL1$\beta$ expression levels ($p = 0.28$).

mRNA expression levels of IL6R and IL1$\beta$ were not associated with PF ($p$-values 0.60–0.64).

3.2.2. Genotype vs. serum hsCRP level
The two studied SNPs in the CRP gene (rs3091244 and rs2794521) were associated with the serum hsCRP level, ($p = 0.02$ and 0.08, respectively). Individuals homozygous for the T allele in the CRP SNP rs3091244 had the highest hsCRP levels (Fig. 2). The other SNPs studied were not associated with serum hsCRP levels ($p$-values 0.49–0.98).

3.2.3. Excluding individuals with depression
Exclusion of the individuals of the part 2 cohort who were depressed at T1 ($N = 25$), resulted in a total of 35 remaining women with PF and 115 never-fatigued women. Neither the SNPs nor the mRNA expression level of IL1$\beta$ and IL6R were found to be associated with the fatigue status in this subset of BCSSs ($p$-values 0.12–0.98 and 0.55–0.89, respectively).

After exclusion of depressed individuals, serum hsCRP levels still differed significantly between those with PF and the never-fatigued subjects ($p = 0.007$). The CRP rs3091244 SNP was borderline significantly associated with serum hsCRP level ($p = 0.06$) in this subset, while the remaining SNPs were not ($p$-values 0.26–0.97).

4. Discussion
Despite the previously demonstrated significant differences in inflammatory biomarkers regarding hsCRP and leukocyte counts among BCSSs with and without fatigue, we could not demonstrate any associations between fatigue and the studied polymorphisms in inflammation-related genes or between fatigue and IL6R or IL1$\beta$ mRNA expression levels. These mRNA expression levels were not associated with IL6R or IL1$\beta$ genotypes. Exclusion of depressed individuals still resulted in non-significant findings. However, in the cohort comprising persistent fatigued and never-fatigued individuals we showed that the CRP SNP rs3091244 [A/G/T] was associated with serum hsCRP levels and that subjects homozygous for the T allele had the highest hsCRP levels.

Prior studies have also demonstrated that the above tri-allelic rs3091244 SNP in the CRP gene affected CRP serum levels with higher levels in individuals carrying the minor T or A alleles (Kathiresan et al., 2006; Hage and Szalai, 2007). This correspondence supports the validity of our findings.

Depression is positively associated with serum CRP levels (Howren et al., 2009), but in our study hsCRP remained significantly associated with fatigue also after exclusion of the depressed individuals. Additionally, BMI is reported to correlate with CRP level (Kathiresan et al., 2006), and fatigued individuals in the current study had higher BMI compared to the non-fatigued individuals. However, even after adjustment for BMI, individuals with PF displayed significantly higher hsCRP compared to those never-fatigued. Thus, increased hsCRP levels seem to remain independently associated with PF.

We did not find that the tested SNPs in the CRP gene were associated with fatigue. CRP is an acute-phase protein which expression is regulated mostly at the transcriptional level, with IL6 being the main inducer and IL1 acting synergistically to enhance the effect (Hage and Szalai, 2007). CRP therefore reflects upstream inflammatory activity (Aukrust et al., 2008). Thus, elevated CRP-values observed in the fatigued women might mirror increased upstream signaling implying that polymorphisms in the CRP gene itself are not necessarily involved in the development of fatigue.

A previous study reported that the presence of cytosine at IL1$\beta$-511 (rs16944) and homozygosity for either variant of the IL6-174 (rs1800795) genotype predicted fatigue in BCSSs (Collado-Hidalgo et al., 2008). The fatigued individuals in that study had significantly higher depression scores compared to those without fatigue. Correcting for depressive symptoms, only a borderline significant relation ($p = 0.052$) between fatigue and the rs16944 SNP in the IL1$\beta$ gene remained (Collado-Hidalgo et al., 2008). Further, after adjustment for age and treatment-related factors, the association between fatigue and the IL6 SNP rs1800795 became non-significant (Collado-Hidalgo et al., 2008). The authors themselves concluded that their results were preliminary due to the small sample size (Collado-Hidalgo et al., 2008). Collado-Hidalgo et al. genotyped 47 women (33 fatigued and 14 non-fatigued) selected from a total of 314 BCSSs who scored $\leq 55$ and $\geq 70$ on the SF-36 vitality subscale, respectively. Due to the use of different fatigue measures, direct comparison between the current and Collado-Hidalgo et al’s study is impeded. However, we genotyped the same SNPs in a much larger cohort of BCSSs, but could not demonstrate any impact of these SNPs on fatigue – even when refining the fatigued vs non-fatigued phenotype in part two. Women recruited by Collado-Hidalgo et al. were diagnosed with early-stage BC (0, I, II), while the BCSSs in the current study had been treated for BC stage II and III. This suggests that women in the current study had received a more uniform and higher treatment burden than those included by Collado-Hidalgo et al. Taken together, the evidence that the IL1$\beta$ rs16944 and the IL6 rs1800795 SNPs are associated with fatigue in women treated for BC stage II and III must be considered relatively weak.

Other genetic alterations in the IL6 gene may be associated with fatigue as supported by recent findings that a SNP in the distal promoter of the IL6 gene (rs4719714) was related to level of fatigue in

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**Fig. 2.** Associations between rs3091244 located in the CRP gene and hsCRP level ($N = 175$). hsCRP levels are log 2 transformed in the figure for optimal illustration. C: cytosine, T: thymine, A: adenine.
cancer patients and their caregivers (Miaskowski et al., 2010). Further, associations between fatigue in lung cancer survivors and two SNPs in the \( IL1\beta \) gene (rs1143633 and rs2853550) were demonstrated (Rausch et al., 2010). Neither the \( IL6 \) rs4719714 nor the \( IL1\beta \) rs1143633 or \( IL1\beta \) rs2853550 SNPs were tested in our study. However, in line with our results which did not support a relation between fatigue and the \( IL6 \) rs1800795 SNP in BCSs, neither did Rausch et al. (2010) find this SNP associated with fatigue in lung cancer survivors.

Two of the SNPs in the \( IL6R \) gene were explored because they had been shown to be associated with serum levels of sIL6R (rs4845617 and rs2228145) (Galicia et al., 2004). The soluble form of IL6R is generated either by proteolytic cleavage from the membrane-bound form of IL6R (mIL6R) or by an alternative mRNA splice variant coding only for the sIL6R (Rose-John et al., 2007). Neither the splice variant coding only for the sIL6R (Rose-John et al., 2007). IL1 demonstrated (Rausch et al., 2010). Neither the mRNA expression level of \( IL6R \) was measured, and information on serum levels of sIL6R was not available, making a direct comparison between the study by Galicia et al. and ours difficult. In addition to the mRNA expression level, also microRNA regulation, post-translational modification and protein degradation are factors decisive for the final levels of a protein. Therefore, our results do not contradict prior findings regarding the relation between the genotypes and the sIL6R levels in serum. In addition to an individual’s genetic background a plethora of environmental factors interplay in the control of mRNA expression and protein level, and the impact of these factors vary in different settings. Our study exploring SNPs in the promoters of the \( IL1\beta \) and \( IL6R \) genes explored only one aspect of this complex process. As our study did not demonstrate any association between the SNPs and mRNA expression levels, probably other factors play a significant role in controlling the expression of these genes in this cohort of BCSs.

The \( IL6R \) SNPs (rs2228145 and rs4129267) have been described to be associated with CRP levels (Ridker et al., 2008). We could not replicate this finding. Compared to the genome-wide association study by Ridker et al. of more than 6000 healthy women, our study comprising 302 individuals must be considered less optimal with respect to demonstrating such associations.

Prior research has focused on the associations between \( IL1\beta \) SNPs and CRP levels (Enquobahrie et al., 2009). Our finding that the CRP SNP rs2794521 was associated with expression levels of \( IL1\beta \) has not been described previously and the results warrant further validation.

Strengths of this study include the large BCSs cohort and the use of the FQ. As fatigue duration ≥6 months was imperative for having CF, individuals with fluctuating fatigue or fatigue of short duration (i.e. acute fatigue) were separated from those with more long-lasting complaints. This should reduce the heterogeneity of the cases. Our study design aimed to achieve a pure as possible fatigue phenotype. The longitudinal design enabled maximization of the phenotypic group differences within the cohort by including only subjects with the highest and lowest fatigue symptom scores (i.e. persistent fatigue vs. never-fatigued individuals). An additional strength is the attempt to further purify the fatigue phenotype by excluding individuals with depressive symptoms.

A limitation of the current, exploratory study is that, based on prior literature and research findings, only seven SNPs related to inflammatory pathways were tested. Therefore, we cannot rule out that other polymorphisms in the same or related genes have an impact on fatigue. As the majority of women included in the study were Norwegian, our conclusions are limited to Caucasian BCSs only. An additional limitation of this study is the small sample sets included in some of the sub-analyses. These results must therefore be confirmed in future studies.

Fatigue is a multi-factorial condition where the mechanisms involved possibly co-vary, both intra- and inter-individually (Barwell and Ancoli-Israel, 2008; Stone and Minton, 2008). We attempted to optimize and refine the phenotypic differences within the study cohort by excluding subjects with depression and transient CF. Still it is possible that other mechanisms alone or in combination with the inflammatory processes were involved in the development of fatigue in the included subjects, thereby bolting our analyses.

5. Conclusion

The seven SNPs explored in the \( IL1\beta, IL6, IL6R \) and \( CRP \) genes as well as the \( IL6R \) and \( IL1\beta \) mRNA expression levels were not associated with fatigue in the investigated BCSs. Neither were SNPs in the \( IL6R \) and \( IL1\beta \) genes associated with mRNA blood expression levels of \( IL6R \) and \( IL1\beta \), respectively. However, the CRP gene SNP rs3091244 was associated with serum hsCRP level among persistent fatigued and never-fatigued BCSs, confirming prior findings. As only a limited number of SNPs was investigated in this study, other polymorphisms in the same or related genes may still impact on fatigue. Therefore, further research should explore this topic using either extensive candidate gene and SNP panels or by performing whole genome analyses.

Conflict of interest

All authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data


References


