ASSOCIATIONS BETWEEN CANDIDATE CYTOKINE GENES AND SLEEP DISTURBANCES AMONG TWO LATENT CLASSES OF BREAST CANCER PATIENTS PRIOR TO AND FOLLOWING TREATMENT

by

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DEDICATION AND ACKNOWLEDGEMENTS

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The text of this thesis is a reprint of the material as it appears in “Associations between candidate cytokine genes and sleep disturbances among two latent classes of breast cancer patients prior to and following treatment” The coauthor listed in this publication directed and supervised the research that forms the basis for the thesis.

ABSTRACT

Objective

The study purposes were to attempt to replicate the association found in our previous study of patients and family caregivers[1] between IL6 and sleep disturbance and to identify additional genetic associations in a larger sample of patients with breast cancer.

Design

Descriptive, longitudinal study

Setting

Patients were recruited from breast care centers located in a Comprehensive Cancer Center, two public hospitals, and four community practices.

Participants

Women (n=398) with breast cancer who had surgery on one breast with no distant metastasis.

Measurements

Questionnaires including the Karnofsky Performance Status (KPS) scale and Self-Administered Comorbidity Questionnaire (SCQ). The 21-item General Sleep Disturbance Scale (GSDS) was used to assess sleep disturbance prior to surgery and monthly for 6 months.

Results

Patients who were younger, had lower KPS scores, and more comorbidities were more likely to be in the high sleep disturbance class. Variations in three cytokine

genes (i.e., Interleukin 13, Nuclear Factor Kappa Beta 2, and Interleukin 1 receptor 2) predicted latent class membership.

Conclusions

Genetic markers may partially explain inter-individual variability among symptom trajectories. Determination of a high risk phenotype and associated genotypes allows for earlier identification of patients at higher risk for developing sleep disturbance and other behavioral symptoms leading to the development of more targeted clinical interventions.

Key words: Sleep disturbance, breast cancer, cytokine genes, growth mixture modeling, latent class, symptom trajectories.

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Introduction

Findings from several studies suggest that women with breast cancer experience a significant amount of sleep disturbance. [2-5] For example, in one large cross sectional study that evaluated prevalence of sleep disturbance in patients with a variety of cancer diagnoses,[2] patients with breast cancer reported the highest rate of insomnia (37.8%) following treatment. In a second study of a heterogeneous sample of oncology patients receiving chemotherapy (CTX),[5] breast cancer patients had the highest rates of insomnia (i.e., 84% reported insomnia symptoms). Of the patients who reported insomnia symptoms, 45% met the diagnostic criteria for insomnia.

Recent work from our research team used growth mixture modeling (GMM) to identify subgroups of patients with distinct sleep disturbance trajectories prior to and for six months following breast cancer surgery.[6] Three distinct latent classes of patients (i.e., high sustained (55.0%), low sustained (39.7%), and decreasing (5.3%)) were identified. Women in the high sustained class were significantly younger, had more comorbidities and poorer functional status, and were more likely to report hot flashes compared to women in the low sustained class. Findings from this study suggest that GMM can be used to identify subgroups of patients with distinct symptom trajectories and distinct phenotypic characteristics.

While an evaluation of differences in phenotypic characteristics is important to determine patients at highest risk for sleep disturbance during and following cancer treatment, an equally important consideration is whether

genomic markers can distinguish among these patient subgroups. As noted by Cirelli[7] specific candidate genes are associated with sleep regulation and sleep disorders like restless leg syndrome[8,9] and narcolepsy. In addition, recent evidence suggests that cytokine dysregulation is associated with sleep disturbance in humans (for reviews see Cirelli[7] and Sehgal and Mignot[10]).

However, only a limited number of studies have evaluated the association between inflammatory cytokines and sleep disturbance. For example, in one study that examined genetic polymorphisms in interleukin 6 (IL6), IL1, and tumor necrosis factor alpha (TNF-A) in patients newly diagnosed with obstructive sleep apnea syndrome (OSAS),[11] the only cytokine gene that was associated with OSAS was a polymorphism located in the promoter of IL6 (C-G-174C, rs1800795). In addition, this association was found only in male patients with OSAS compared to unaffected males. A higher percentage of male patients with OSAS (35.1%) were homozygous for the minor C allele compared to males in the control group (10.3%; p=.004). Recent work from our team found an association between IL6 rs35610689 and self-reported sleep disturbance in patients and family caregivers prior to and following radiation treatment. Minor allele carriers had a decreased odds of belonging to the higher sleep disturbance class (p=.006).[1]

The purpose of the current study, using the sample of patients with breast cancer described previously,[6] was to attempt to replicate the association between IL6 and sleep disturbance found in our previous study of patients and family caregivers[1] and to identify additional associations in a larger sample. To

achieve this objective, we evaluated for differences in phenotypic and genotypic characteristics between breast cancer patients who were classified into the high sustained (58.1%) and low sustained (41.9%) GMM classes (Figure 1). Patients in the decreasing class were not included in this analysis because the sample size (n= 21) was too small to allow for meaningful comparisons among the three latent classes.

MATERIALS AND METHODS

Patients and Settings

This analysis is part of a larger study that evaluated neuropathic pain and lymphedema in women who underwent breast cancer surgery. Patients were recruited from breast care centers located in a Comprehensive Cancer Center, two public hospitals, and four community practices.

Patients were eligible to participate if they: were an adult woman (≥18 years) who underwent breast cancer surgery on one breast; were able to read, write, and understand English; agreed to participate; and gave written informed consent. Patients were excluded if they were having breast cancer surgery on both breasts and/or had distant metastasis at the time of diagnosis.

A total of 516 patients were approached to participate, 410 were enrolled (response rate 79.4%), and 398 completed the baseline assessment. The most common reasons for refusal were: too busy, overwhelmed with the cancer diagnosis, or insufficient time available to do the baseline assessment prior to surgery.

Instruments

The demographic questionnaire obtained information on age, marital status, education, ethnicity, employment status, and living situation. Karnofsky Performance Status (KPS) scale is widely used to evaluate functional status in patients with cancer and has well established validity and reliability.[12,13] Patients rated their functional status using the KPS scale that ranged from 30 (I feel severely disabled and need to be hospitalized) to 100 (I feel normal; I have no complaints or symptoms).

Self-Administered Comorbidity Questionnaire (SCQ) is a short and easily understood instrument that was developed to measure comorbidity in clinical and health service research settings.[14] The questionnaire consists of 13 common medical conditions that were simplified into language that could be understood without any prior medical knowledge. Patients were asked to indicate if they had the condition using a “yes/no” format. If they indicated that they had a condition, they were asked if they received treatment for it (yes/no; proxy for disease severity) and did it limit their activities (yes/no; indication of functional limitations). Patients were given the option to add two additional conditions not listed on the instrument. For each condition, a patient can receive a maximum of 3 points. Because the SCQ contains 13 defined medical conditions and 2 optional conditions, the maximum score totals 45 points if the open-ended items are used and 39 points if only the closed-ended items are used. The SCQ has well-established validity and reliability and has been used in studies of patients with a variety of chronic conditions.[15,16]

The 21-item General Sleep Disturbance Scale (GSDS) was used to evaluate overall sleep disturbance during the past week. Each item is rated on a scale that ranges from 0 (never) to 7 (everyday). The total GSDS score can range from 0 (no disturbance) to 147 (extreme sleep disturbance). A total GSDS score of ≥43 indicates a clinically meaningful level of sleep disturbance.[17] Cronbach's alpha for the GSDS total score was 0.86.

Study Procedures

The study was approved by the Committee on Human Research at the University of California, San Francisco and by the Institutional Review Boards at each of the study sites. During the patient's preoperative visit, a clinician explained the study and determined the patient's willingness to participate. For those women who were willing to participate, the clinician introduced the patient to the research nurse. The research nurse met with the women, determined eligibility, and obtained written informed consent prior to surgery. After obtaining consent, patients completed the enrollment questionnaires on average 4 days prior to surgery. Medical records were reviewed for disease and treatment information.

Genomic analyses

Gene selection - Cytokines and their receptors are classes of polypeptides that mediate inflammatory processes.[18] Cytokine dysregulation is associated with sleep disturbance.[7,10] These polypeptides are divided into pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines promote systemic inflammation and include: interferon gamma (IFNG), IFNG 1 receptor (IFNGR1),

IL1R1, IL2, IL8, IL17A, nuclear factor kappa beta (NFKB1), NFKB2, and TNF.[18,19] Anti-inflammatory cytokines suppress the activity of pro-inflammatory cytokines and include: IL1R2, IL4, IL10, and IL13.[18,19] Of note, IFNG1, IL1B, and IL6 possess pro- and anti-inflammatory functions.[19]

Blood collection and genotyping - Of the 398 patients who completed the baseline assessment, 310 provided a blood sample from which DNA could be isolated from peripheral blood mononuclear cells (PBMCs). No differences were found in any demographic and clinical characteristics between patients who did and did not choose to participate in the study or in those patients who did and did not provide a blood sample for genomic analyses.

Genomic DNA was extracted from PBMCs using the PUREGene DNA Isolation System (Invitrogen, Carlsbad, CA). DNA was quantitated with a Nanodrop Spectrophotometer (ND-1000) and normalized to a concentration of 50 ng/μL (diluted in 10 mM Tris/1 mM EDTA). Genotyping was performed blinded to clinical status and positive and negative controls were included. Samples were genotyped using the Golden Gate genotyping platform (Illumina, San Diego, CA) and processed according to the standard protocol using GenomeStudio (Illumina, San Diego, CA). Two blinded reviewers visually inspected signal intensity profiles and resulting genotype calls for each SNP. Disagreements were adjudicated by a third reviewer. If consensus could not be reached, the SNP was excluded.

SNP selection - A combination of tagging SNPs and literature driven SNPs (i.e., reported as being associated with altered function and/or symptoms) were selected for analysis. Tagging SNPs were required to be common (defined as

having a minor allele frequency ≥0.05) in public databases (e.g., HapMap). In order to ensure robust genetic association analyses, quality control filtering of SNPs was performed. SNPs with call rates <95%, or Hardy-Weinberg p<.001 were excluded. As shown in Table 1, a total of 103 SNPs among the 15 candidate genes (IFNG1: 6 SNPs, IFNGR1: 1SNP; IL1B: 12 SNPs; IL1R1: 5 SNPs; IL1R2: 3 SNPs; IL2: 5 SNPs; IL4: 9 SNPs; IL6: 12 SNPs; IL8: 3 SNPs; IL10: 8 SNPs; IL13: 5 SNPs; IL17A: 6 SNPs; NFKB1: 14 SNPs; NFKB2: 4 SNPs; TNF: 10 SNPs) passed all quality control filters and were included in the genetic association analyses. Potential functional roles of SNPs associated with sleep disturbance were examined using PUPASuite 2.0 [20] a comprehensive search engine that tests a series of functional effects (i.e., non-synonymous changes, altered transcription factor binding sites, exonic splicing enhancing or silencing, splice site alterations, microRNA target alterations).

Statistical Analyses for the Phenotypic Data

Data were analyzed using SPSS version 19 (SPSS, Chicago, IL) and STATA Version 9.[31] Descriptive statistics and frequency distributions were generated for sample characteristics. Independent sample t-tests (for continuous variables), Mann-Whitney U tests (for continuous variables not normally distributed), and Chi square analyses (for categorical variables) were used to evaluate for differences in demographic and clinical characteristics between the two latent classes. All calculations used actual values. Adjustments were not made for missing data. Therefore, the cohort for each analysis was dependent on the largest set of available data between groups.

Unconditional GMM with robust maximum likelihood estimation was carried out to identify latent classes with distinct sleep disturbance trajectories. These methods are described in detail elsewhere.[6,21] In brief, a single growth curve that represented the “average” change trajectory was estimated for the whole sample. Then, the number of latent growth classes that best fit the data was identified using guidelines recommended by a number of experts.[22-24]

Model fit for the GMM was assessed statistically by identifying the model with the lowest Bayesian Information Criterion (BIC). The parametric bootstrapped likelihood ratio test (BLRT) was used to evaluate whether a model with K classes fit the data better than a model with K-1 classes. In addition to using the BLRT to compare models, we examined the Vuong-Lo-Mendell-Rubin Likelihood Ratio Test (VLMR) for the “K” versus “K-1” class models. When the VLMR test is non-significant, it does provide evidence that the K-class model is not better than the K-1-class model. The fourth index used to evaluate model fit was entropy, with >.80 being preferred.[25,26] Finally, the best fitting model was visually inspected by plotting observed against model-predicted values to determine whether the predicted trajectories followed the empiric trajectories for the classes and to evaluate whether the predicted plots “made sense” theoretically and clinically.[27]

Intercepts and linear and quadratic slopes for each latent class were estimated for each model. Intercept variances were estimated for each class and were allowed to differ across classes. Given the relatively small sample sizes, the within-class linear and quadratic slope variances were fixed at zero for two

classes, because estimation failed when they were free to vary. Without setting these slope variances to zero, the model could not be estimated due to non-positive definite covariance matrices. Mixture models are known to produce solutions at local maxima, so each model was fit with random starts to be sure that the solution for the model with the maximum log likelihood values was replicated [26] Missing data for the sleep disturbance scores were accommodated by Mplus Version 5.21 through the use of Full Information Maximum Likelihood and the use of the Expectation-Maximization algorithm. This method assumes that any missing data are missing at random[28]

Statistical Analyses for the Genetic Data

Allele and genotype frequencies were determined by gene counting. Hardy-Weinberg equilibrium was assessed by the Chi-square or Fisher Exact tests. Measures of linkage disequilibrium (i.e., D' and r^2) were computed from the participants' genotypes with Haploview 4.2. Linkage disequilibrium (LD)-based haplotype block definition was based on D' confidence interval.[29]

For SNPs that were members of the same haploblock, haplotype analyses were conducted in order to localize the association signal within each gene and to determine if haplotypes improved the strength of the association with the phenotype. Haplotypes were constructed using the program PHASE version 2.1.[32] In order to improve the stability of haplotype inference, the haplotype construction procedure was repeated 5 times using different seed numbers with each cycle. Only haplotypes that were inferred with probability estimates of $\geq$.85, across the five iterations, were retained for downstream analyses. Only inferred

haplotypes that occurred with a frequency estimate of ≥15% were included in the association analyses, assuming a dosage model (i.e., analogous to the additive model).

For association tests, three genetic models were assessed for each SNP: additive, dominant, and recessive. Barring trivial improvements (i.e., delta <10%), the genetic model that best fit the data, by maximizing the significance of the p-value was selected for each SNP. Logistic regression analysis that controlled for significant covariates as well as race/ethnicity, was used to evaluate the association between genotype and sleep disturbance group membership. Only those genetic associations identified as significant from the univariate analyses were evaluated in the multivariate analyses. A backwards stepwise approach was used to create the most parsimonious model. Except for race/ethnicity, only predictors with a p-value of <.05 were retained in the final model. Genetic model fit and both unadjusted and covariate-adjusted odds ratios were estimated using STATA version 9.[31]

Based on the recommendations of Rothman,[32] adjustments were not made for multiple testing. However, rigorous controls were imposed on the analysis of the SNPs with p-values of <.05. As described above, each of these SNPs was evaluated using logistic regression analyses that controlled for differences in phenotypic characteristics, as well as potential confounding due to population stratification. Only those SNPs that remained significant were included in the final presentation of the results. In addition, the actual number of independent tests is more appropriately considered in relationship to the total

number of cytokine genes evaluated (n=15), because the majority of the SNPs within each gene locus were in LD. Therefore, the finding of three significant independent associations (2 SNPs and one haplotype) is unlikely to be due solely to chance. Findings are reported for all of the SNPs that were evaluated to have these data available in the literature for subsequent comparisons and meta-analyses (see Table 1).

Ancestry informative markers (AIMs) can be used as a tool to minimize confounding due to population stratification in case-control association studies.[34-36] Homogeneity in ancestry among participants was verified by principal component analysis,[37] using HelixTree (GoldenHelix, Bozeman, MT). Briefly, the number of principal components (PCs) was sought which distinguished the major racial/ethnic groups in the sample by visual inspection of scatter plots of orthogonal PCs (i.e., PC 1 versus PC2, PC2 versus PC3). This procedure was repeated until no discernable clustering of patients by their self-reported race/ethnicity was possible (data not shown). The first three PCs were selected to adjust for potential confounding due to population substructure (i.e., race/ethnicity) by including them in all logistic regression models. One hundred and six AIMs were included in the analysis.

Results

Differences in Demographic and Clinical Characteristics

As summarized in Table 2, no differences were found between the low sustained and high sustained sleep disturbance classes for the majority of the demographic and clinical characteristics. However, patients in the high sustained

class were significantly younger, had a lower KPS score, and a higher number of comorbidities (all p <.0001). In addition, a lower percentage of patients in the high sustained class were employed (p=.04) and a higher percentage had received CTX during the first 6 months after breast cancer surgery (p=.02).

Candidate gene analyses of the two GMM classes – As summarized in Table 1, the minor allele frequency was significantly different between the two latent classes for 5 SNPs and one haplotype: IL1R2 haplotype (Hap) A2, IL6 rs35610689, IL10 rs1878672, IL13 rs1881457, IL13 rs1800925, and NFKB2 rs1056890. For IL6 rs35610689 (p=.037) and IL10 rs1878672 (p=.043), a recessive model fit the data best. For IL13 rs1881457 (p=.011), IL13 rs1800925 (p=.002), and NFKB2 rs1056890 (p=.025), a dominant model fit the data best. The IL1R2 haplotype (composed of rs11674595-rs757041) was found to be significantly different between the two latent classes (p=.037).

Regression analyses of IL1R2, IL13, and NFKB2 genotypes and lower versus higher sleep disturbance classes

In order to better estimate the magnitude (i.e., odds ratio, OR) and precision (95% confidence interval, CI) of genotype on the odds of belonging in the higher sleep disturbance class as compared to the lower sleep disturbance class, multivariate logistic regression models were fit. In addition to genotype, the phenotypic variables evaluated in the model were age (5 year increments), working for pay, number of comorbid conditions, functional status (estimated by the KPS total score, in 10 point increments), receiving CTX in the six months following breast cancer surgery, and having undergone a sentinel node biopsy

(SLNB). After adjusting for age, functional status, number of comorbid conditions, receiving CTX in the six months following breast cancer surgery, and having undergone a SLNB, only working for pay was not retained in the final models.

The only genetic associations that remained significant in the multivariate logistic regression analyses were for IL1R2 Hap A2 (composed of rs11674595-rs757041), IL13 rs1800925, and NFKB2 rs1056890 (Table 3). In the regression analysis for IL1R2 Hap A2, after controlling for race/ethnicity, genotype, age, functional status, number of comorbid conditions, receiving CTX in the six months following breast cancer surgery, and having undergone a SLNB were the variables retained in the final model ($p<.0001$). As shown in Figure 2, Hap A2 is composed of alleles at two SNPs (i.e., rs11674595 [T major allele], rs7570441 [A minor allele]). The overall model explained 15.5% of the variance in sleep disturbance class membership. Controlling for age, functional status, number of comorbid conditions, receiving CTX in the six months following breast cancer surgery, and having undergone a SLNB, each additional dose of IL1R2 Hap A2 was associated with 2.08-fold increased odds of belonging to the higher sleep disturbance class ($p=.024$).

In the regression analysis for IL13 rs1800925 (see Figure 3A), after controlling for race/ethnicity; genotype, age, functional status, number of comorbid conditions, receiving CTX in the six months following breast cancer surgery, and having undergone a SLNB were the predictors retained in the final model ($p<.0001$). The overall model explained 16.2% of the variance in sleep disturbance class membership. Controlling for age, functional status, number of

comorbid conditions, receiving CTX in the six months following breast cancer surgery, and having undergone a SLNB, carrying one or two doses of the minor allele (i.e., CC versus CT + TT) was associated with a 2.21-fold increase in the odds of belonging in the higher sleep disturbance class (p=.005).

In the regression analysis for NFKB2 rs1056890 (see Figure 3B), after controlling for race/ethnicity; genotype, age, functional status, number of comorbid conditions, receiving CTX in the six months following breast cancer surgery, and having undergone a SLNB were the predictors retained in the final model (p<.0001). The overall model explained 15.4% of the variance in sleep disturbance class membership. Controlling for age, functional status, number of comorbid conditions, receiving CTX in the six months following surgery for breast cancer, and having undergone a SLNB, carrying one or two doses of the minor allele (i.e., CC versus CT + TT) was associated with 47% decrease in the odds of belonging to the higher sleep disturbance class (p=.028).

Discussion

This study is the first to evaluate the effects of a number of phenotypic characteristics and variations in cytokine genes on sleep disturbance in breast cancer patients following surgery. Of note and consistent with previous reports of patients with breast cancer, patients in the high sustained class were younger,[2] had lower KPS scores, and had more comorbidities.[38] In addition, a lower percentage of women in the high sustained class were working for pay and a higher percentage received CTX.

It is interesting to note that the phenotypic characteristics that distinguished the high sustained class in our current study are consistent with our previous study of oncology patients and FCs[1]. In both studies, participants in the high sustained class were younger, had poorer functional status, and reported a higher number of comorbidities. These findings suggest that these characteristics place individuals at higher risk for sleep disturbance and need to be part of clinicians' risk assessment for this significant clinical problem.

A primary aim of this study was to replicate the genetic associations identified in our previous study. Of note, the association between NFKB2 observed in our previous study was found in the current study. In our previous study,[1] carrying one or two doses of the minor allele for NFKB2 rs7897947 was associated with a 74% decrease in the odds of belonging to the higher sleep disturbance class (p=.022). In the current study, an association was found in the same cytokine gene, but with a different SNP (NFKB2 rs1056890, p=.025).Variations in NFKB2 rs1056890 in the current study explained 1.3% of the variance in sleep disturbance class membership. Women who carried one or two doses of the minor "T" allele had a 47% decrease in their odds of belonging to the higher sleep disturbance class. While the SNPs in NFKB2 were different, in both studies carriers of the minor allele were less likely to be classified in the higher sleep disturbance class. The differences in the SNP associations identified may be related to differences in sample size and/or phenotypic characteristics (e.g., gender) between the two studies. Finally, the SNPs

identified in our prior (rs1056890) and current (rs7897947) study, may be in linkage disequilibrium with an unmeasured causal SNP(s).

NFKB2 is a pro-inflammatory cytokine that belongs to the nuclear factor-Kappa Beta family that is made up of transcription factors that regulate various biological processes including immunity, stress responses, apoptosis, and cellular differentiation.[39] Inappropriate activation of NFKB has been linked to inflammatory processes such as autoimmune arthritis, asthma, lung fibrosis, and septic shock.[39] Prior to our recent work,[1] polymorphisms in NFKB2 have not been linked directly to sleep disturbance in oncology patients or their family caregivers. The SNP identified in our previous study (i.e., NFKB2 rs7897947) is located in the intron. The SNP identified in the current study (NFKB2 rs1056890) is located in the promoter. Findings from our two studies support a role for NFKB2 in the inflammatory process that may be involved in the development of sleep disturbance.

In this study, variation in IL13 rs1800925 explained 2.2% of the variance in sleep disturbance class membership. Carrying one or two doses of the minor “T” allele was associated with a 2.21-fold increase in the odds of belonging to the higher sleep disturbance class. This SNP is located in the intron of IL13 and has no known function. While polymorphisms in IL13 have not been linked with sleep disturbance, variations in IL13 are associated with a number of inflammatory processes including asthma and eczema.[41] One study [40] described an association between IL13 rs1800925 and psoriasis. Why this association was not

identified in our previous study[1] warrants investigation in future studies with larger samples of oncology patients and family caregivers.

The third association identified in this study was between sleep disturbance and the IL1R2 Haplotype A2 (Hap A2) that is composed of two SNPs (i.e., rs11674595, rs7570441). Variations in IL1R2 Haplotype A2 explained 1.3% of the variance in sleep disturbance class membership. Each additional dose of IL1R2 Haplotype A2 was associated with a 2.08 increase in the odds of belonging to the higher sleep disturbance class. Prior to this study, no associations were found between this haplotype and sleep disturbance. However in another study from our research team,[21] a different haplotype in the same region (i.e., a 3-SNP haplotype composed of the rare "C" allele of rs4141134, the common "T" allele of rs11674595, and the rare "A" allele of rs7570441) was associated with an increased odds (OR=2.10, 95% CI 1.117,3.959, p=.021) of belonging to the class with a higher level of depressive symptoms. This haplotype explained 1.9% of the variance in depressive symptom latent class membership.

While the functions of each of the individual SNPs in the haplotype are not known, two of the SNPs (rs11674595 and rs7570441) are located in introns. IL1R2 is an anti-inflammatory cytokine that blocks inflammatory signaling and inhibits pro-inflammatory IL1 activity by acting as a decoy receptor.[43] Therefore, IL1R2 plays a role in the regulation of inflammatory pathways and its association with sleep disturbance requires further study.

Lastly, we did not replicate an association found in our previous study between IL6 and sleep disturbance. In our prior study,[1] IL6 rs35610689 and a number if covariates explained 13.4% of the variance in sleep disturbance class membership. Carrying one or two doses of the minor allele (i.e., AG+GG) was associated with a 78% decrease in the odds of belonging to the higher sleep disturbance class (MAF=.242; p=.004). In our current study although an association between IL6 rs35610689 and sleep disturbance group membership was observed it did not remain significant after adjusting for covariates. Taken together the associations between IL6 and sleep disturbance warrant further study.

Limitations

Although our sample size was adequate, future studies with larger sample sizes need to be conducted in the same population in order to confirm these findings and identify additional latent classes and/or covariates. Differences in demographic and clinical characteristics of the participants between this study and previous studies[1,6] may partially explain why we did not replicate all of our findings. In the current study, the sample consisted entirely of females with breast cancer, while in our previous study oncology patients with various cancer diagnoses as well as males and females were included. Some phenotypic characteristics were similar between the two samples, which may explain our ability to partially replicate the findings from our previous study. While, the genotypic findings were somewhat consistent, additional investigations of candidate genes and sleep disturbance are warranted.

Despite these limitations, these findings provide evidence to support distinct sleep disturbance phenotypes in breast cancer patients prior to and following surgery. The higher risk phenotype has been associated with other symptoms such as depression and fatigue. It is important that these patients be identified early in order to better treat their symptoms and intervene early in the trajectory. Our findings of genetic associations between previously studied and new cytokine genes provide evidence to support a role for inflammation in the development and maintenance of sleep disturbance and other behavioral symptoms in oncology patients.

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Figure legends

Figure 1 - Observed and estimated General Sleep Disturbance Scale (GSDS) trajectories for participants in each of the latent classes, as well as the mean GSDS scores for the total sample.

Figure 2- To be developed

Figure 3A Differences between the latent classes in the percentages of participants who were homozygous for the common allele (CC) or heterozygous or homozygous for the minor allele (CT+TT) for rs1800925 in interleukin 13 (IL13).

Figure 3B Differences between the latent classes in the percentages of participants who were homozygous for the common allele (CC) or heterozygous or homozygous for the minor allele (CT+TT) for rs1056890 in nuclear factor kappa beta 2 (NFKB2).

Table 1. Summary of Single nucleotide Polymorphisms Analyzed for Pro- and Anti-Inflammatory Cytokine Genes and the Growth Mixture Model Analysis for General Sleep Disturbance Total Score

Gene	SNP	Position	Chr	MAF	Alleles	Chi Square	p-value	Model
IFNG1	rs2069728	66834051	12	.079	G>A	.737	.692	A
IFNG1	rs2069727	66834490	12	.411	A>G	.729	.694	A
IFNG1	rs2069718	66836429	12	.442	C>T	.454	.797	A
IFNG1	rs1861493	66837463	12	.264	A>G	1.866	.393	A
IFNG1	rs1861494	66837676	12	.279	T>C	1.892	.388	A
IFNG1	rs2069709	66839970	12	.008	G>T	FE	1.000	A
IFNG1	HapA3					1.837	.399	
IFNG1	HapA5					.812	.666	
IFNGR1	rs9376268	137574444	6	.246	G>A	.597	.742	A
IL1B	rs1071676	106042060	2	.189	G>C	.859	.651	A
IL1B	rs1143643	106042929	2	.383	G>A	.086	.958	A
IL1B	rs1143642	106043180	2	.082	C>T	3.049	.218	A
IL1B	rs1143634	106045017	2	.187	C>T	.871	.647	A
IL1B	rs1143633	106045094	2	.392	G>A	.283	.868	A
IL1B	rs1143630	106046282	2	.115	C>A	3.951	.139	A
IL1B	rs3917356	106046990	2	.450	A>G	.355	.837	A
IL1B	rs1143629	106048145	2	.389	T>C	1.749	.417	A
IL1B	rs1143627	106049014	2	.397	T>C	1.828	.401	A
IL1B	rs16944	106049494	2	.386	G>A	2.266	.322	A
IL1B	rs1143623	106050452	2	.277	G>C	.021	.990	A
IL1B	rs13032029	106055022	2	.448	C>T	.809	.667	A
IL1B	HapA1					.379	.827	
IL1B	HapA4					.106	.948	
IL1B	HapA6					.875	.645	
IL1B	HapB1					1.452	.484	
IL1B	HapB6					.301	.860	
IL1B	HapB8					.427	.808	
IL1R1	rs949963	96533648	2	.223	G>A	3.293	.193	A
IL1R1	rs2228139	96545511	2	.053	C>G	2.659	.265	A
IL1R1	rs3917320	96556738	2	.047	A>C	4.308	.116	A
IL1R1	rs2110726	96558145	2	.317	C>T	1.118	.572	A
IL1R1	rs3917332	96560387	2	.187	T>A	1.514	.469	A
IL1R1	HapA1					.059	.971	
IL1R1	HapA2					.329	.848	
IL1R1	HapA3					1.426	.490	
IL1R2	rs4141134	96370336	2	.362	T>C	.011	.995	A
IL1R2	rs11674595	96374804	2	.247	T>C	.993	.609	A
IL1R2	rs7570441	96380807	2	.408	G>A	1.357	.507	A
IL1R2	HapA1					1.572	.456	
IL1R2	**HapA2**					**FE**	**.037**	
IL1R2	HapA4						.762	
IL2	rs1479923	119096993	4	.308	C>T	.391	.822	A
IL2	rs2069776	119098582	4	*.184*	T>C	n/a	n/a	n/a
IL2	rs2069772	119099739	4	.241	A>G	.222	.895	A
IL2	rs2069777	119103043	4	.047	C>T	.159	.924	A
IL2	rs2069763	119104088	4	.277	T>G	1.565	.457	A

IL2	HapA1					.580	.748	
IL2	HapA2					1.613	.446	
IL2	HapA3					.222	.895	
IL4	rs2243248	127200946	5	.086	T>G	2.604	.272	A
IL4	**rs2243250**	**127201455**	**5**	***.269***	**C>T**	**12.018**	**.002**	**A**
IL4	**rs2070874**	**127202011**	**5**	***.245***	**C>T**	**7.109**	**.029**	**A**
IL4	rs2227284	127205027	5	*.387*	C>A	2.418	.298	A
IL4	rs2227282	127205481	5	*.390*	C>G	2.002	.367	A
IL4	rs2243263	127205601	5	.124	G>C	1.503	.472	A
IL4	rs2243266	127206091	5	*.237*	G>A	5.170	.075	A
IL4	rs2243267	127206188	5	*.237*	G>C	5.417	.067	A
IL4	**rs2243274**	**127207134**	**5**	***.261***	**G>A**	**7.699**	**.021**	**A**
IL4	HapA1					.390	.823	
IL4	HapA3					.454	.797	
IL4	HapX1					2.939	.230	
IL6	rs4719714	22643793	7	.255	A>T	3.053	.217	A
IL6	rs2069827	22648536	7	.069	G>T	4.431	.109	A
IL6	rs1800796	22649326	7	*.134*	G>C	.849	.654	A
IL6	rs1800795	22649725	7	.285	C>G	1.583	.453	A
IL6	rs2069835	22650951	7	*.130*	T>C	2.596	.273	A
IL6	rs2066992	22651329	7	.091	G>T	.889	.641	A
IL6	rs2069840	22651652	7	.333	C>G	4.107	.128	A
IL6	rs1554606	22651787	7	.319	T>G	.187	.911	A
IL6	rs2069845	22653229	7	.319	G>A	.187	.911	A
IL6	rs2069849	22654236	7	.024	C>T	1.998	.368	A
IL6	rs2069861	22654734	7	.056	C>T	2.473	.290	A
IL6	**rs35610689**	**22656903**	**7**	**.259**	**A>G**	**FE**	**.037**	**R**
IL6	HapA1					1.280	.527	
IL6	HapA5					3.623	.163	
IL6	HapA8					1.471	.479	
IL8	rs4073	70417508	4	.455	T>A	.888	.642	A
IL8	rs2227306	70418539	4	.366	C>T	2.088	.352	A
IL8	rs2227543	70419394	4	.368	C>T	1.847	.397	A
IL8	HapA1					.888	.642	
IL8	HapA4					1.903	.386	
IL10	rs3024505	177638230	1	.129	C>T	1.987	.370	A
IL10	rs3024498	177639855	1	.204	A>G	.259	.878	A
IL10	rs3024496	177640190	1	.421	T>C	5.439	.066	A
IL10	**rs1878672**	**177642039**	**1**	**.416**	**G>C**	**FE**	**.043**	**R**
IL10	rs3024492	177642438	1	*.161*	A>T	.062	.969	A
IL10	rs1518111	177642971	1	.303	G>A	2.561	.278	A
IL10	rs1518110	177643187	1	.301	G>T	2.213	.331	A
IL10	rs3024491	177643372	1	.408	T>G	4.998	.082	A
IL10	HapA1					2.493	.287	
IL10	HapA2					2.930	.231	
IL10	HapA8					.329	.849	
IL13	**rs1881457**	**127184713**	**5**	**.210**	**A>C**	**FE**	**.011**	**D**
IL13	**rs1800925**	**127185113**	**5**	**.233**	**C>T**	**FE**	**.002**	**D**
IL13	rs2069743	127185579	5	.019	A>G	.917	.632	A
IL13	rs1295686	127188147	5	.265	G>A	2.902	.234	A
IL13	rs20541	127188268	5	.212	C>T	1.010	.604	A

IL13	HapA1					3.247	.197	
IL13	HapA4					.727	.695	
IL17A	rs4711998	51881422	6	.346	G>A	2.290	.318	A
IL17A	rs8193036	51881562	6	.327	T>C	4.927	.085	A
IL17A	rs3819024	51881855	6	.372	A>G	1.002	.606	A
IL17A	rs2275913	51882102	6	.361	G>A	2.172	.338	A
IL17A	rs3804513	51884266	6	.023	A>T	FE	.055	A
IL17A	rs7747909	51885318	6	.217	G>A	1.470	.479	A
NFKB1	rs3774933	103645369	4	.409	T>C	2.139	.343	A
NFKB1	rs170731	103667933	4	.397	T>A	1.457	.483	A
NFKB1	rs17032779	103685279	4	.023	T>C	FE	.462	A
NFKB1	rs230510	103695201	4	.366	T>A	.249	.883	A
NFKB1	rs230494	103706005	4	.477	A>G	.772	.680	A
NFKB1	rs4648016	103708706	4	.017	C>T	FE	.226	A
NFKB1	rs4648018	103709236	4	.025	G>C	FE	.759	A
NFKB1	rs3774956	103727564	4	.479	C>T	.502	.778	A
NFKB1	rs10489114	103730426	4	.025	A>G	FE	.759	A
NFKB1	rs4648068	103737343	4	.366	A>G	.752	.687	A
NFKB1	rs4648095	103746914	4	.052	T>C	FE	1.000	A
NFKB1	rs4648110	103752867	4	.205	T>A	2.612	.271	A
NFKB1	rs4648135	103755716	4	.060	A>G	FE	1.000	A
NFKB1	rs4648141	103755947	4	.188	G>A	4.570	.102	A
NFKB1	rs1609798	103756488	4	.337	C>T	1.531	.465	A
NFKB1	HapA1					.156	.925	
NFKB1	HapA9					1.261	.532	
NFKB2	rs12772374	104146901	10	.157	A>G	1.312	.519	A
NFKB2	rs7897947	104147701	10	.229	T>G	.554	.758	A
NFKB2	rs11574849	104149686	10	.085	G>A	1.360	.507	A
NFKB2	**rs1056890**	**104152760**	**10**	**.317**	**C>T**	**FE**	**.025**	**D**
TNFA	rs2857602	31533378	6	.341	T>C	.864	.649	A
TNFA	rs1800683	31540071	6	.390	G>A	.119	.942	A
TNFA	rs2239704	31540141	6	.335	G>T	1.020	.601	A
TNFA	rs2229094	31540556	6	.278	T>C	1.562	.458	A
TNFA	rs1041981	31540784	6	.386	C>A	.076	.963	A
TNFA	rs1799964	31542308	6	.224	T>C	4.349	.114	A
TNFA	rs1800750	31542963	6	.016	G>A	FE	.250	A
TNFA	rs1800629	31543031	6	.149	G>A	1.219	.544	A
TNFA	rs1800610	31543827	6	.100	C>T	1.258	.533	A
TNFA	rs3093662	31544189	6	.074	A>G	2.396	.302	A
TNFA	HapA1					3.187	.203	
TNFA	HapA5					2.905	.234	
TNFA	HapA6					2.526	.283	

A = additive model, Chr = chromosome, D = dominant model, IFNG = interferon gamma, IL = interleukin, MAF = minor allele frequency, n/a = not assayed because SNP violated Hardy-Weinberg expectations ($p<0.001$), NFKB = nuclear factor kappa beta, R = recessive model, SNP= single nucleotide polymorphism, TNFA = tumor necrosis factor alpha

Single nucleotide polymorphisms (SNPs) that violated Hardy-Weinberg expectations are denoted in italics in the MAF column.

Table 2. Differences in Demographic and Clinical Characteristics Between Low (n=158) and High (n=219) Sustained Sleep Disturbance Groups

Characteristic	Low Sustained n=158 (41.9%) Mean (SD)	High Sustained n=219 (58.1%) Mean (SD)	Statistic and p-value
Age (years)	57.7 (12.1)	53.0 (10.9)	t=3.93, p<0.0001
Education (years)	15.5 (2.6)	15.9 (2.7)	t=1.38, p=0.18
Karnofsky Performance Status score	96.5 (6.8)	90.9 (11.7)	t=5.76, p<0.0001
Self-administered Comorbidity Questionnaire score	3.7 (2.4)	4.8 (3.1)	t=-3.86, p<0.0001
Total number of breast biopsies in the past year	1.53 (0.879)	1.48 (0.757)	U, p=0.90
	N (%)	N (%)	FE
Ethnicity			
White	102 (65)	136 (62.4)	
Black	11 (7.0)	28 (12.8)	p=0.23
Asian/Pacific Islander	24 (15.3)	24 (11.0)	
Hispanic/Mixed ethnic background/Other	20 (12.7)	30 (13.8)	
Married/partnered (% yes)	62 (39.5)	92 (42.6)	p=0.60
Lives alone (% yes)	37 (23.7)	50 (23.1)	p=0.90
Working for pay (% yes)	86 (54.4)	93 (43.1)	p=0.04
Stage of disease at diagnosis			
0	25 (15.8)	40 (18.3)	
I	72 (45.6)	74 (33.8)	
IIA	32 (20.3)	61 (27.9)	U, p=0.34
IIB	18 (11.4)	23 (10.5)	
IIIA	5 (3.2)	14 (6.4)	
IIIB	2 (1.3)	1 (0.5)	
IIIC	3 (1.9)	6 (2.7)	
IV	1 (0.6)	0 (0.0)	
Type of Surgery			
Breast Conservation	131 (82.9)	177 (80.8)	p=0.69
Mastectomy	27 (17.1)	42 (19.2)	
Sentinel node biopsy (% yes)	138 (87.3)	174 (79.5)	p=0.053
Axillary lymph node dissection (% yes)	52 (32.9)	92 (42.2)	p=0.07
Breast reconstruction at time of surgery (% yes)	30 (19.1)	45 (20.5)	p=0.79
Neoadjuvant chemotherapy (% yes)	27 (17.1%)	48 (22.0)	p=0.30
Radiation therapy during first 6 months (% yes)	99 (62.7%)	117 (53.4)	p=0.09
Chemotherapy during first 6 months (% yes)	43 (27.2)	86 (39.3)	p=0.02

Abbreviations: FE = Fisher's Exact, SD = standard deviation.

Table 3. Multiple Logistic Regression Analyses for IL1R2, IL13, and NFKB2 candidate gene markers

Predictor	Odds Ratio	Standard Error	95% CI	Z	p-value
IL1R2 haplotype	2.08	0.673	1.101, 3.921	2.26	0.024
age	0.85	0.055	0.747, 0.964	-2.52	0.012
KPS	0.52	0.095	0.362, 0.744	-3.57	<0.001
No. comorbids	1.18	0.069	1.048, 1.320	2.75	0.006
Chemotherapy	2.43	0.745	1.330, 4.427	2.89	0.004
Sentinel node biopsy	0.31	0.126	0.141, 0.690	-2.88	0.004
Overall model fit: $\chi^2 = 60.40$, $p < .0001$ $R^2 = 0.1548$					
IL13 Genotype	2.21	0.619	1.277, 3.827	2.83	0.005
Age	0.85	0.056	0.743, 0.963	-2.54	0.011
KPS	0.55	0.098	0.384, 0.776	-3.37	0.001
No. comorbids	1.17	0.070	1.036, 1.311	2.54	0.011
Chemotherapy	2.19	0.670	1.203, 3.987	2.56	0.010
Sentinel node biopsy	0.38	0.152	0.171, 0.829	-2.42	0.015
Overall model fit: $\chi^2 = 63.34$, $p < .0001$ $R^2 = 0.1624$					
NFKB2 Genotype	0.53	0.152	0.306, 0.935	-2.19	0.028
Age	0.84	0.056	0.739, 0.958	-2.61	0.009
KPS	0.54	0.098	0.378, 0.769	-3.41	0.001
No. comorbids	1.16	0.070	1.034, 1.309	2.51	0.012
Chemotherapy	2.32	0.706	1.274, 4.208	2.75	0.006
Sentinel node biopsy	0.35	0.140	0.161, 0.769	-2.62	0.009
Overall model fit: $\chi^2 = 60.05$, $p < .0001$ $R^2 = 0.1539$					

Multiple logistic regression analysis of candidate gene associations with lower versus higher GSDS GMM groups. For each model, the first three principal components identified from the analysis of ancestry informative markers as well as self-report race/ethnicity were retained in all models to adjust for potential confounding due to race or ethnicity (data not shown). Predictors evaluated in each model included genotype (IL1R2 haplotype A2 composed of rs4141134-rs11674595-rs7570441: zero, one, or two doses of the C-T-G); IL13 rs1800925: CC versus CT + TT; NFKB2 rs1056890: CC versus CT + TT), age (in 5 year increments), and functional status at baseline (estimated by the KPS score, 10 point increments), number of comorbid conditions, receiving chemotherapy in the six months following surgery for breast cancer, and having undergone a sentinel node biopsy.

Abbreviations; CI =confidence interval; GMM = Growth Mixture Model; GSDS = General Sleep Disturbance Scale; IL13 = interleukin 13; IL1R2= interleukin 1 receptor 2; KPS = Karnofsky Performance Status; NFKB2 = nuclear factor kappa beta 2.

Figure 1

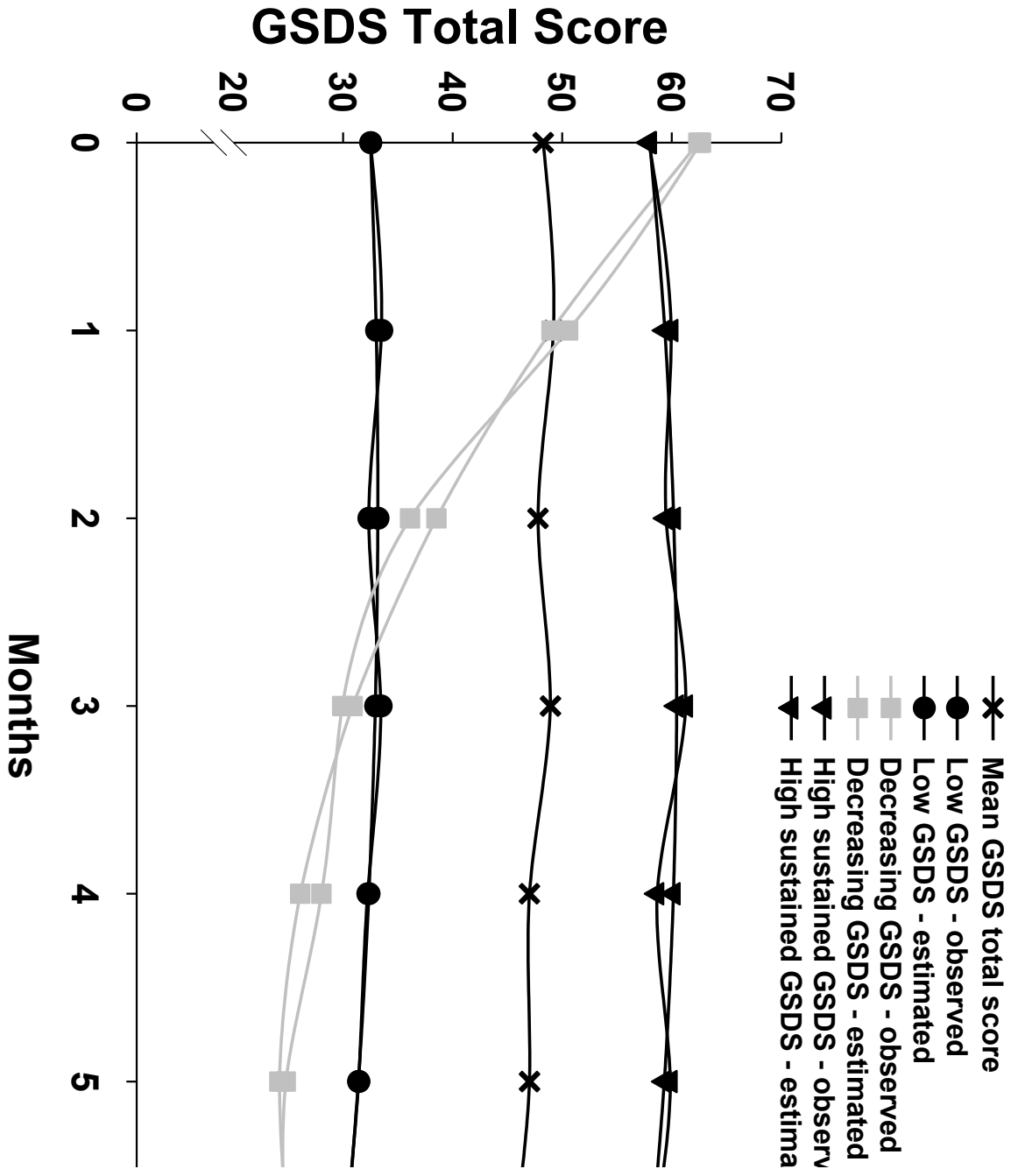
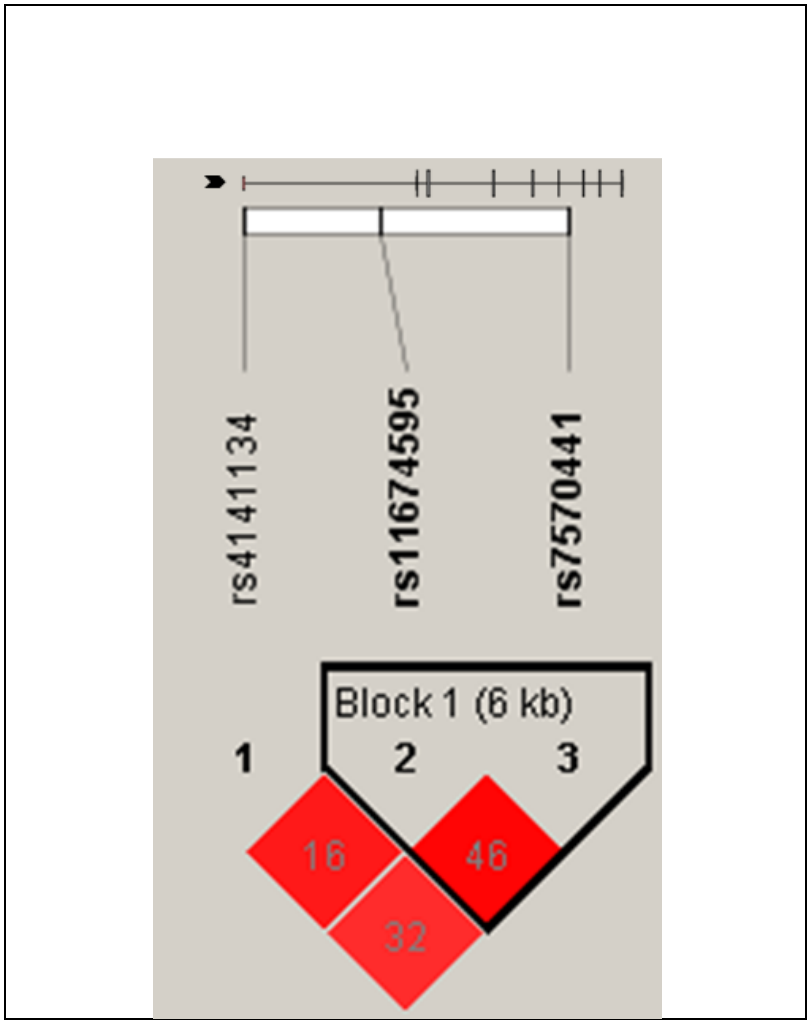
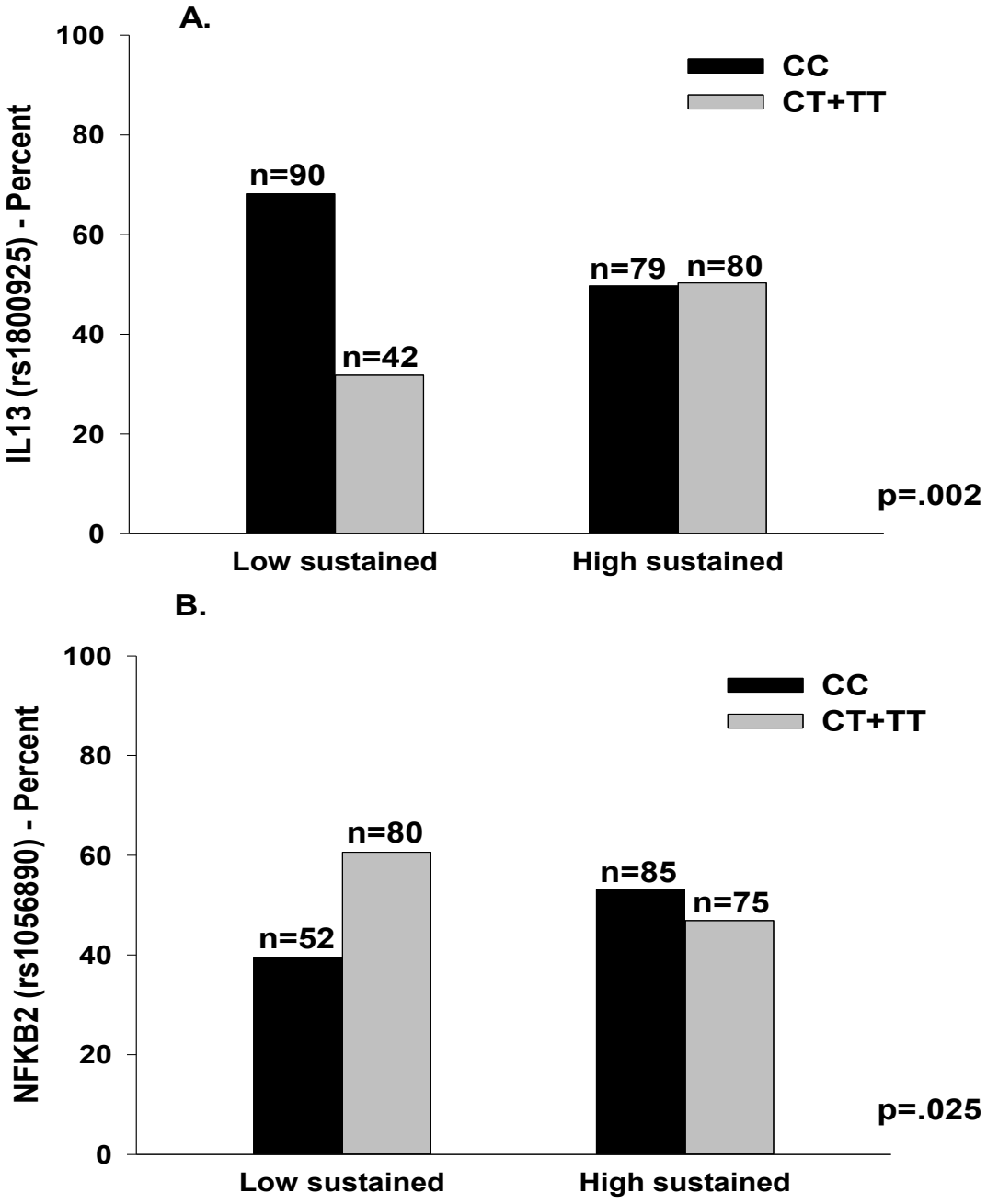

GSDS Total Score
0
20
30
40
50
60
70
0
1
2
3
4
5
Months
Mean GSDS total score
Low GSDS - observed
Low GSDS - estimated
Decreasing GSDS - observed
Decreasing GSDS - estimated
High sustained GSDS - observ
High sustained GSDS - estima

Figure 2



Haplotype	Low Sustained	High Sustained
A1: T-G	157 (59.5%)	183 (68.3%)
A2: T-A	***29 (11.0%)***	***1 (0.0%)***
A3: C-G	1 (0.0%)	0 (0.0%)
A4: C-A	77 (29.2%)	84 (31.3%)

Figure 3


A.
IL13 (rs1800925) - Percent
100
80
60
40
20
0
CC
CT+TT
n=90
n=42
n=79
n=80
p=.002
Low sustained
High sustained
B.
NFKB2 (rs1056890) - Percent
100
80
60
40
20
0
CC
CT+TT
n=52
n=80
n=85
n=75
p=.025
Low sustained
High sustained

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