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# CRHR1 and CRHR2 pre-treatment gene expression may predict malignant melanoma patients neuropsychiatric responses to IL-2 therapy

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

CRHR1 and CRHR2 pre-treatment gene expression may predict malignant melanoma patients' neuropsychiatric responses to IL-2 therapy

By Caitlin N. Barbarita

Although, Interleukin-2 (IL-2) immunotherapy has been shown to be successful in initiating tumor regression in up to 20% of malignant melanoma patients, it induces an array of toxicities that hinder effective treatment. Corticotropin Releasing Hormone receptors 1 and 2 (CRHR1 and CRHR2, respectively) expression on the pituitary are known to affect the HPA axis, the functioning of which is believed to be altered in these patients. The following study examines the relationship between CRHR1 and CRHR2 pre-treatment expression in patient blood samples and patients' neuropsychiatric responses to IL-2 therapy.

After providing informed consent, 20 patients with Stage IV melanoma eligible for IL-2 immunotherapy were recruited. Prior to, during, and after IL-2 administration we measured genetic expression of CRHR1 and CRHR2, plasma concentrations of ACTH and IL-6, neurobehavioral symptoms, and tolerance of IL-2 treatment. A neurobehavioral analysis was performed utilizing two symptom complexes, mood and neurovegetative. Non-parametric statistics were used to examine changes in biological and behavioral variables. Spearman's correlation and simple linear regression determined relationships between CRHR1 and CRHR2 gene expression, IL-2 dose tolerance, and neurobehavioral symptoms.

Linear regression models were statistically insignificant; however we did see a statistically significant positive partial correlation between IL-2 doses tolerated and CRHR1 gene expression within the model. This small study illustrates the need for further understanding of the mechanisms of symptom development and treatment response during IL-2 immunotherapy.

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# CRHR1 and CRHR2 pre-treatment gene expression may predict malignant melanoma patients' neuropsychiatric responses to IL-2 therapy

## *IL-2 Immunotherapy*

Cytokines, a vast family of regulatory proteins involved in different levels of signaling within the immune system, are critical not only for activation of host immune defense, but also alter sleep, appetite, and psychomotor activity. The cytokine interleukin-2 (IL-2), acts as a growth factor for natural killer cells and T and B cells of the immune system (Warise et al., 2008). High dose IL-2 therapy has also been effective in treating stage IV malignant melanoma, one of the most resistant forms of cancer. Although early detection and treatment of melanoma vastly increases the patients' outcome, about a third of early stage cases of melanoma will metastasize. Still, the one year survival rate of treated metastic melanoma patients is only 25% (Senzer et al., 2009).

An important aspect of tumorigenesis is the ability of the cancer cells to evade rejection from the body's own immune system. The immune system and cancer cells share a complex relationship. Both activation and suppression of the immune system provides a welcoming environment for growing tumors as activation releases a number of pro-tumorigenic factors and suppression increases the tumors ability to evade the immune system (Muller et al., 2005). Despite this complex relationship, a systemic review done in 2007 on the efficacy of IL-2 therapy for malignant melanoma patients found that although it had a fairly low tumor regression rate of less than 20%, it increased average life expectancy of the stage IV patient to 70 months and is currently the standard of care in both the United States and Canada (Petrella et al., 2006).

Although high-dose IL-2 may be the best option for stage IV melanoma patients, it has a high toxicity rate and during IL-2 treatment patients are prone to gastrointestinal,

cardiovascular, pulmonary, renal, hepatic, septic and neurologic toxicity. Also common among immunotherapy patients is the incidence of neuropsychiatric side effects such as depression and psychosis. According to a study conducted by Musselman et al. in 2001, another immunotherapeutic drug used to treat earlier stages of melanoma, Interferon-α, induces major depression in 45% of malignant melanoma patients undergoing IFN- α treatment. The interesting relationship between neuropsychiatric symptoms and the immune system is strengthened by the finding that plasma levels of IL-6 are significantly higher in melanoma patients undergoing immunotherapy and diagnosed with major depression compared to those cancer patients undergoing immunotherapy without major depression (Musselman et al., 2001). These detrimental side effects, particularly the neurological toxicity in the form of depression and psychosis, interfere with the continuation of this potentially life-saving therapy. These complications of immunotherapy are most likely caused by the activation of "secondary" cytokines through stimulation of both the T-cells and B-cells of the immune system (Nicholson, 2006).

According to a review by Capuron et al. 2004, immune-induced sickness behavior manifests most obviously in behavioral changes including "anhedonia, cognitive dysfunction, anxiety/irritability, psychomotor slowing, fatigue, anorexia, sleep alterations and increased sensitivity to pain" (p 819). These symptoms of sickness behavior have been reproduced in healthy animals through injections of a number of cytokines including IL-1, IL-2, IL-6 and TNF-α and by administering agents that induce the proinflammatory cytokine chain (Capuron et al., 2004).

*IL-2 Immunotherapy and pro-inflammatory cytokines*

Two different pathways, both stimulated by IL-2, are believed to be responsible for the neurological side effects of this treatment. The first pathway begins with the IL-2-induced release of the cytokine, Interferon-gamma (IFN–γ). IFN–γ is a pro-inflammatory cytokine associated with tumor regression. Once released from the T-cells, IFN-γ activates the enzyme Indoleamine dioxygenase (IDO). Once activated, IDO initiates the catabolism of the amino acid tryptophan. Tryptophan is essential for the synthesis of many different proteins and the catabolism initiated by IFN-γ results in a local depletion of tryptophan. With the depletion of tryptophan, replication of intracellular viruses and uncontrolled proliferating cells are disrupted. Thus, it is believed that the anti-tumor effects of IFN-γ are attributable to this consequent tryptophan depletion (Widner et al., 2000).

However, elevated levels of IFN-γ and the consequent depletion of tryptophan may also be involved in neurotoxic and neuropsychiatric side effects of IL-2 treatment in malignant melanoma patients. Tryptophan is essential for the production of the neurotransmitter serotonin and thus constant activation of IDO would result in a decreased production of serotonin. IFN-γ is also involved more directly in the depletion of serotonin by initiating the transcription of the 5HT reuptake transporter. Depletion of serotonin has been associated with depression and psychiatric disorders as well as the modulation of pain in the spinal cord. Studies have demonstrated that irritability and pain are two of the most common side effects of tryptophan depletion (Menkes et al., 2000). In 2002, Huang et al. found that that decreased serum tryptophan in colorectal cancer patients significantly correlated with a decrease in the quality of life scores of those

patients. Although these patients did not undergo immunotherapy, the immune system, and more specifically IFN-γ, was activated by a tumor–related mechanism (Huang et al., 2002).

Tryptophan's metabolite, kynurenine, breaks down further into two compounds, quinolinic acid and kynurenic acid. These two compounds have been shown to directly alter neuronal firing and cause neurotoxic effects. Quinolinic acid is a glutamate NMDA receptor agonist, causing increased neuronal firing. Kynurenic acid has been found to be neuroprotective as it is a glutamate receptor antagonist. However, it seems it may be involved in the pathogenesis of schizophrenia and the appearance of psychotic symptoms as according to Erhardt et al. 2007.

*HPA Axis and IL-2 Immunotherapy*

The second pathway, and the focus of this research is IL-2-induced changes on hypothalamic-pituitary-adrenal (HPA) axis functioning. The HPA axis is a network of activation and feedback inhibition between the hypothalamus, pituitary and adrenal gland. This axis has been show to influence stress, digestion, the immune system, emotion, sex drive and fatigue. The HPA axis network begins at the hypothalamus which releases Corticotropin Releasing Hormone (CRH). CRH then acts on CRHR1 and CRHR2 receptors on the anterior pituitary and initiates the release of Adrenocorticotropic Hormone (ACTH). ACTH acts on the adrenal cortex. The adrenal cortex releases cortisol which, in turn, inhibits the release of more ACTH and CRH through receptors on the hypothalamus and anterior pituitary. Cortisol also inhibits the production of the cytokines IL-1 and IL-6. However, three cytokines, TNF-α, IL-1, and IL-6 act on both the

hypothalamus and the pituitary and stimulate the production of CRH and ACTH (Pariante, 2009).

In addition to inhibiting further release of ACTH, CRH, IL-6 and IL-1, cortisol is

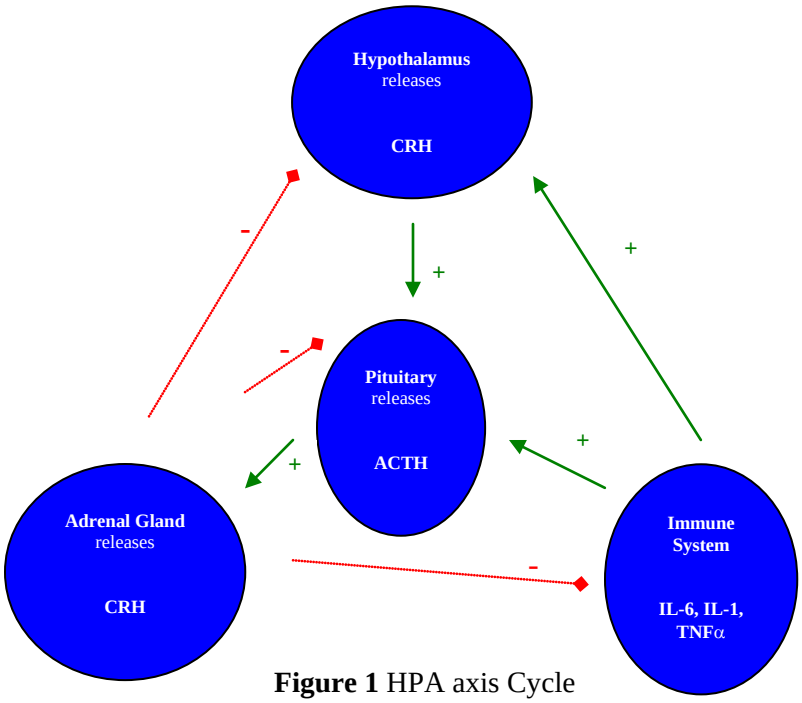


**Figure 1** HPA axis Cycle

also involved in integral processes within the body. Cortisol is involved in the regulation of blood pressure, the immune response, glucose metabolism, inflammatory response and insulin release. Cortisol is also known to induce a stress response that allows for increased concentration, increased blood pressure and other symptoms central to the "fight or flight" response.

Despite cortisol's integral role in the proper functioning of the body, chronically elevated levels of cortisol, through hyperactivity of the HPA axis, can have very damaging effects. Chronically high levels of cortisol have been associated with cognitive

disturbances, affective syndrome, psychotic symptoms, suppressed neurogenesis, metabolic syndrome and most notably depression (Kloet et al., 2007).

Hyperactivity of the HPA axis has long been associated with major depression. In as early as 1956, Board et al. found that the vast majority of patients with major depressive disorder had elevated levels of plasma cortisol. Denikoff et al. found that in a population of 44 metastatic patients treated with IL-2 combined with Lymphokine-Activated Killer cells, 15 experienced "severe behavioral changes that required acute intervention" and "22 experienced severe cognitive changes" (p 293) (Denicoff et al., 1987). In 2001, Musselman et al. reported that malignant melanoma patients undergoing high dose IFN-α treatment, another pro-inflammatory cytokine immunotherapy, with no previous history of depression or psychiatric disorders who experienced elevated levels of both ACTH and cortisol during the first round of IFN-α treatment had a greater risk of developing depression later on in treatment (Musselman et al., 2001).

These cancer patients with co-morbid depression undergoing immunotherapy also show the inability to suppress the production of cortisol and ACTH in a dexamethasone test (Musselman et al., 2001). Nonsuppression during this test is a common effect seen in major depressive disorders as well as psychoses. Normal healthy controls are able to suppress 85% of cortisol and ACTH production when administered dexamethasone. However, patients with major depression are generally able to suppress only 45% of cortisol and ACTH production. This test shows the inability to regulate the HPA axis through its normal feedback inhibition is most reasonably the underlying cause of the hyperactivity of the HPA axis and overproduction of cortisol (Pariante, 2009). The fact that immunotherapy patients also show this deficiency points towards a more prominent

role for pro-inflammatory cytokines in the morphology of psychiatric illnesses. Previous studies as well as studies within this lab (see preliminary data section) have found an association between elevated levels of cortisol and ACTH and the pro-inflammatory cytokine IL-6 both in patients with major depressive disorder and in patients undergoing IL-2 therapy (Capuron et al., 2001, Pariante, 2009).

*CRH Receptors 1 & 2 and IL-2 Immunotherapy*

Although the incidence of neuropsychiatric side effects from IL-2 treatment is a large barrier to effective treatment, not every patient is afflicted with these symptoms. Many patients are able to successfully complete all four cycles of IL-2 therapy with minimal adverse events, while others must drop out after the first cycle. However, there may be a way to predict patients' neuropsychiatric responses to IL-2 treatment. In her paper, Pariante shows lines of evidence that HPA axis hyperactivity not only occurs during "acute phases of a psychiatric illness" but it is highly likely that it also predicts the risk of developing such a disorder (Pariante, 2009). We predict that the same will be true in malignant melanoma patients undergoing IL-2 treatment. We hypothesize that Corticotropin Releasing Hormone Receptors 1 and 2 (CRHR1 and CRHR2, respectively) expression pre IL-2 therapy may be able to predict a patient's neuropsychiatric response to IL-2 therapy.

CRH receptors 1 and 2 are transmembrane, G-Protein coupled receptors that mediate the physiological actions of CRH and CRH-like peptides, such as urocortin. Although CRH receptors are most commonly known for their action on the pituitary and their expression is usually measured within brain in animals studies, a study by Hsuchou

et al. in 2009 found that CRHR1 and CRHR2 mRNA do in fact cross the blood-brain barrier. This allows us the opportunity to include the blood measurement of CRH receptor expression within this clinical study.

Though CRH receptors share many common biological characteristics, studies have shown that CRHR1 binds CRH with a higher affinity than CRHR2 suggesting that the two receptor types probably mediate different physiological responses. It has been suggested that, due to the different binding affinity, CRHR1 may to be responsible for mediating a more normal response to stress and an anxiogenic effect. CRHR1 may also be involved in depressogenic actions of the HPA axis and CRHR1 antagonists have been proposed to treat both chronic anxiety disorder and depression. CRHR2, on the other hand, seems to mediate both an anxiolytic and anorexogenic response and CRHR2 antagonists have been proposed to treat chronic pain and fatigue syndromes (Grammatopoulos et al., 2002).

*Symptom Complexes in Immunotherapy*

In 2005, Capuron et al. found, in a study using IFN-a, that two categories of symptoms, neurovegetative and mood, are most likely induced by two different pathways. This was shown by splitting up questions from the HAM-D, HAM-A and NRS into the two different categories. Capuron et al. hypothesized that at least two methods may be responsible for the behavioral symptoms seen in IFN-α patients; “1) activation of corticotropin-releasing factor (CRF) pathways; and 2) depletion of relevant monoamines” (p 820) (Capuron et al., 2004). However, due to the different actions mediated by each receptor, we believe that the CRH receptors may play a role in the incidence of the

different symptoms. A neurobehavioral analysis will be done utilizing the two symptom complexes, mood and neurovegetative. As in Capuron et al., 2004, these symptom complexes will be assessed using the following interviews/questionnaires; the Hamilton Anxiety Scale, the Hamilton Depression Scale and the Neurotoxicity Rating Scale.

The HAM-A is a 14-item structured interview designed to quantify the severity of anxiety symptomology. Each item is defined by a series of symptoms, and measures both psychic anxiety (mental agitation and psychological distress) and somatic anxiety (physical complaints related to anxiety). Each item is rated on a 5-point scale, ranging from 0 (not present) to 4 (severe). Scores range from 0 to 56, with a score of 14 indicating clinically significant anxiety (Hamilton 1959).

The HAM-D is a 21-item structured interview designed to measure the severity of depressive symptoms in adults. The HAM-D is one of the most widely used instruments for measuring outcome in mood disorders. The HAM-D contains items that assess: somatic symptoms, insomnia, working capacity and interest, mood, guilt, psychomotor retardation, agitation, anxiety, and insight. Each question has between 3-5 possible responses, which increase in severity. A score of 0 to 6 indicates a normal state; a score of 7 to 17 indicates mild depression; a score of 18 to 24 indicates moderate depression; and a score of 25 or higher indicates severe depression (Hamilton 1960).

The Neurotoxicity rating scale is a self-reported rating test consisting of 38 symptoms associated with neurotoxicity. Incidence and severity of each symptom is ranked by the patients as 0, indicating symptom not present, to 4, indicating symptom extremely severe (Capuron et al., 2003).

We hypothesize that CRHR1 and CRHR2 pre-treatment gene expression and the symptom complex scores during Cycle 1 and Cycle 3 will predict IL-2 dose tolerated. We will examine both Cycle 1 and Cycle 3 separately for this relationship. We also plan to examine relationship between the immune system activation as determined by IL-6 plasma concentrations and the CRHR1 and CRHR2 gene expression.

## Predictions

We predict that IL-2 doses tolerated will decrease by each Cycle, with Cycle 1 having the highest average doses tolerated and Cycle 4 having the least average doses tolerated. We think that each symptom complex average will increase each day within both Cycle 1 and Cycle 3 with an overall peak occurring at Cycle 3 day 3. However, we predict the effects of IL-2 treatment to be additive and as such we will see more significant change in Cycle 3 compared to Cycle 1.

We predict that increased CRHR1 pre-treatment gene expression will result in an increase in the mood symptom complex scores and a decrease in doses tolerated for Cycles 1 and 3. Conversely we predict that increased CRHR2 pre-treatment gene expression will result in an increase in the neurovegetative symptom complex scores and a decrease in doses tolerated for Cycles 1 and 3.

We expect that our study sample size will decrease as treatment continues, and those patients with lower CRHR1 and CRHR2 gene expression will receive the most IL-2 doses in Cycle 1 and Cycle 3 treatment. We think that the IL-2 treatment will have no significant effect on both CRHR1 and CRHR2 expression, thus there will be no significant difference between the pre-treatment expression and the post-treatment

expression. Lastly, we predict that expression of both CRHR1 and CRHR2 pre-treatment will correlate with levels of ACTH and IL-6, supporting the HPA axis model described earlier.

## Methods

### *Clinical Procedures*

Non-depressed patients with stage IV malignant melanoma, ages 18 through 75, were recruited from the Emory Winship Cancer Institute and were offered participation in a double-blind, randomized controlled trial (escitalopram vs. placebo). Convenience sampling methodology was used in the recruitment of patients. After informed consent was obtained, study patients received treatment with either the anti-depressant escitalopram or placebo 1-2 weeks prior to beginning intravenous IL-2 therapy [720,000 units/kg Q8 hours X 5 days (1 Cycle) every 3 weeks X 4 Cycles]. Although each Cycle lasted five days, data was collected from patients for only the first three days for patient comfort and to enhance compliance. For the purposes of the parent study, patients cannot be un-blinded for the analyses; therefore patient data were analyzed as one group over time. Each patient undergoes cancer restaging after Cycle 2 of IL-2 treatment. If the cancer has progressed, the patient is discontinued from IL-2 and the study.

Blood draws were performed at regular intervals just prior to and during each IL-2 Cycle. Plasma ACTH, cortisol and IL-6 levels were measured via radioimmunoassay according to the manufacturer's protocol (Nichols Institute, ICN Biomedicals, and R&D systems respectively). IL-2 treatment adherence was evaluated according to the patient's medical records. Severity of depression was measured with the HAM-D, whereas

severity of anxiety was measured with the HAM-A and severity of neurotoxic symptomology was measured using the Neurotoxicity Rating scale. All data were entered into a SPSS database version 17.0 (© 2010 SPSS Inc.). The study was approved by both the Emory University Institutional Review Board and the Winship Cancer Center Clinical Trials Office.

*Genetic Laboratory Methodology*

A total of thirty samples were collected into a Blood RNA PAXgene™ RNA tube 1-2 weeks before patient was set to begin IL-2 treatment. The blood RNA tube is a 2.76 mL of additive per mL of blood. After blood is introduced into the tube, the intracellular RNA profile remains stable for 6 months at -70/-80°C. The total RNA was extracted using PAXgene™ Blood RNA kit. Samples are collected again about 4 weeks post IL-2 treatment for those patients who completed a follow-up visit.

Once the RNA was extracted from the blood sample, it was placed in the -80°C freezer. The RNA samples remain stable at -80°C for up to 50 months. The RNA samples were then taken to the Emory Biomarker Service Center Core Lab to determine the expression of the CRHR1 and CRHR2 genes through Taqman (ABI) qRT-PCR. First the biocore lab performed a purity assessment on the RNA samples using spectrophotometer. The assessment was performed using two different UV ratios; 260/280 ratio and 260/230 ratio. Next, the quantity of RNA within each sample was performed using Quant-iT RiboGreen. Both of these first two assessments were done to ensure reliable results.

The kit used for the Reverse Transcription step was the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Catalog number: 4368813). All kit

components were stored at -15° to -25° C to ensure optimum quality. The total RNA samples were checked to make sure they were free of inhibitors of reverse transcription and PCR, dissolved in PCR-compatible buffer or water and free of RNase activity to ensure optimal performance of the cDNA reverse transcription reaction. Samples were prepared and the Reverse Transcription was run following the manufacturer's protocol. Once completed the samples were stored at 2° to 6° C or -15° to -25° C before undergoing the PCR reaction (Applied Biosystems 2006).

The reagent used for PCR step was the TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Catalog number: 4324018). The control gene used in comparison to the CRHR1 and CRHR2 genes was the GUSB-VIC gene. PCR was performed according to the manufactor's protocol and the quantity of probes and primers was determined using the spectrophotometer. The PCR Cycle at which the Rn value exponentially increases (Ct) was determined for both the control gene and the experimental genes. Delta Ct was determined by subtracting the Ct value for the control gene from the experimental genes Ct values. The Delta Ct values were used for analysis (TaqMan® 2002) (See Figure 2).

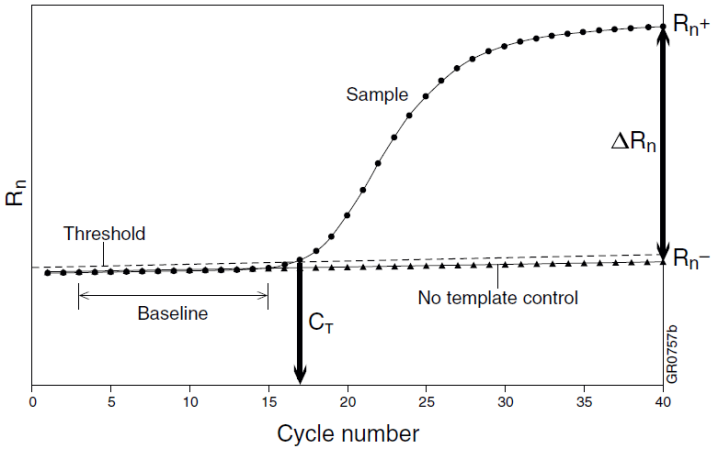


**Figure 2** Graph describing how gene expression is measured using RT-PCR. Ct value is Cycle at which sample begins to exponentially fluoresce due to replication. (TaqMan® 2002)

*Biostatisical Methodology*

As stated previously, for the purposes of the parent study, patients cannot be un-blinded for the analyses. Therefore, patient data was analyzed as one group over time. All analyses were done using the statistical program SPSS version 17.0 (© 2010 SPSS Inc.).

Symptom Complexes were determined using previous guideline proven successful by Capuron et al. 2005 and Musselman et al. 2009. Symptoms were split into two main categories; mood and neurovegetative. Within each category symptoms were split further into two different subcategories. The Mood Complex contained depressive symptoms and anxious symptoms while the Neurovegetative Complex contained vegetative and somatic symptoms. A breakdown of which questions from the HAM-A, HAM-D and NRS were used to determine each category can be found below in Table 1.

Depressive scores were determined by the HAM-D questions about depressed mood, feelings of guilt, suicidal thoughts, and a lack of interest in work and activities (anhedonia**)**. The anxious scores were determined by the HAM-A questions about anxious mood, tension, and phobias. Vegetative scores were determined by the HAM-D question about observer rated decreased motor activity, and the NRS questions difficulty getting to sleep, difficulty staying asleep, sleeping too much, loss of appetite, and tiredness/fatigue. Somatic scores were determined by NRS questions all-over sick feeling, nausea, vomiting, body aches, joint pain, and other pain.

**Table 1** Outline of methods used to determine the four symptoms complexes

| Symptom Complexes | | |
|---|---|---|
| Mood Complex | Depressive | Average of HAM-D questions 1-3 and 7 |
| | Anxious | Average of HAM-A questions 1-3 |
| Neurovegetative Complex | Vegetative | Average of HAM-D question 8 and NRS questions 10-12, 15-16 |
| | Somatic | Average of NRS questions 9, 13-14 and 18-20 |

First, the demographics of the population of patients were assessed using descriptive statistics. Differences in categorical demographic variables were assessed using the Chi-square test. Continuous demographic variables were described using mean and standard deviations. The Shapiro-Wilks test was used to to dictate whether parametric or non-parametric methods should be utilized to examine the following variables: gene expression, IL-2 dose tolerance, and each of the symptom complexes.

The Shapiro-Wilks test showed that both gene expression and IL-2 dose tolerance are normally distributed in this sample, thus a parametric paired samples t-test was used to determine any significant change in these two variables. The Shapiro-Wilks test showed symptom complexes are not normally distributed in this sample, thus the non-parametric Friedman test and Wilcoxon rank test were used to determine any significant changes in each measure between the sampling time points.

Due to the skewed distribution of the symptom complex data, 0.5 was added to all symptom complex values and a log transformation was performed so that a linear regression analysis could be performed to assess the relationship between IL-2 doses tolerated and CRHR1 and CRHR2 genetic expression for Cycle 1 and Cycle 3. We chose to examine only Cycle 1 and 3 for the following reasons: 1) Cycle 1 has the most participants (n=20), 2) Patients are restaged just prior to Cycle 3 leaving those patients whose cancer has not progressed for analysis, and 3) Patients in Cycle 3 exhibit the highest biological markers and behavioral scores. Levels of IL-6, ACTH in Cycle 1 and Cycle 3 and pre-treatment gene expression were assessed using the non-parametric Spearman's correlation to determine the relationship between these variables.

## Results

*Study Patient Demographics*

Of the 23 patients who provided informed consent, 20 patients were included in this study. All twenty patients had RNA samples drawn pre-treatment and ten patients had samples drawn post treatment. Post-treatment RNA samples were not collected from patients if they withdrew from the study or their condition worsened.

Thirty-five percent of patients completed all four Cycles of IL-2 treatment, 20% of patients dropped from the study following Cycle 3, 30% of patients dropped following Cycle 2 and 15% of patients dropped after the first treatment Cycle. There are no statistically significant differences in the number of patients in each Cycle. The average age of our patients was 47 and all but one patient was enrolled with a diagnosis of Stage 4 melanoma. There was a significant difference with respect to gender in our study sample, 75% were male ($p=0.025$). Fifty-five percent of our patients had a partial college educational background, while only 25% were college graduates ($p=0.011$). Thirty percent of our patients made $100,000 a year or above and most patients were married at the time of enrollment in the study ($p=<0.001$). Lastly, 50% of our patients were initially diagnosed with cancer two to five years prior to enrollment in this study. The mean HAM-D and HAM-A were both 6 indicating that patients entered the study with no significant anxious or depressive symptoms.

The mean total number of IL-2 doses received throughout therapy was 19, with the maximum number of doses possible being 60. The average number of IL-2 doses tolerated for Cycle 1 was 9. For both Cycle 2 and Cycle 3 the average dose tolerated was

6. For Cycle 4, the average dose tolerated was 4. The maximum dose possible per Cycle was 15 (See Table 2 and Figure 3).

**Table 2** Demographics of the study sample

| **Characteristic** | | **N Value** | **Percentage** | **p-value*** |
|---|---|---|---|---|
| **Gender** | | | | 0.025** |
| | Male | 15 | 75% | |
| | Female | 5 | 25% | |
| **Education** | | | | 0.011** |
| | High School | 3 | 15% | |
| | Partial college | 11 | 55% | |
| | college graduate | 5 | 25% | |
| | Graduate school | 1 | 5% | |
| **Income** | | | | 0.359 |
| | less than $20,000 | 2 | 10% | |
| | $20-$39,999 | 1 | 5% | |
| | $40-$59,999 | 3 | 15% | |
| | $60-$79,999 | 4 | 20% | |
| | $80-$99,999 | 3 | 15% | |
| | $100,000 or above | 6 | 30% | |
| | Refuse | 1 | 5% | |
| **Relationship** | | | | <0.001** |
| | Single | 2 | 10% | |
| | Married | 17 | 85% | |
| | Divorced | 1 | 5% | |
| **Time Since Cancer Diagnosis** | | | | 0.157 |
| | within 1 year | 7 | 35% | |
| | 2-5 years | 10 | 50% | |
| | 6 or more years ago | 3 | 15% | |
| **Racial Identity** | | | | <0.001** |
| | African-American | 1 | 5% | |
| | Asian | 1 | 5% | |
| | Caucasian | 18 | 90% | |
| **Stage of Cancer** | | | | <0.001** |
| | Stage 3 | 1 | 5% | |
| | Stage 4 | 19 | 95% | |
| **Age** | | 47+/-13.252 | N/A | N/A |
| **HAM-D Screening** | | 6+/-4.247 | N/A | N/A |
| **HAM-A Screening** | | 6+/-3.773 | N/A | N/A |
| **Total number of IL-2 doses received** | | 19+/-8.439 | N/A | N/A |

*Chi-square test used to determine p-values
**Indicates significance

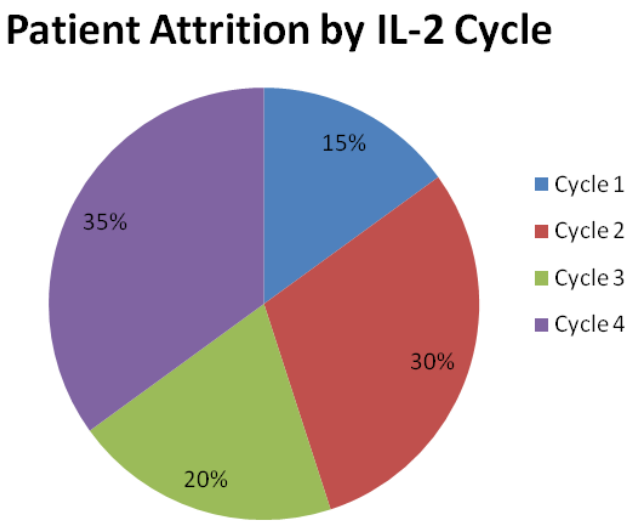


**Figure 3** Patient attrition throughout treatment by IL-2 Cycle. 15% withdrew after Cycle 1, 30% withdrew after Cycle 2, 20% withdrew after Cycle 3 and 35% completed all 4 cycles of IL- treatment

*Changes in IL-2 Dose Tolerated*

The IL-2 doses tolerated for each Cycle showed a normal distribution using the Shapiro-Wilk test. A parametric paired t-test showed that the average number of doses tolerated by the patients does change significantly between Cycles. The first and greatest significant change in dose tolerated is between Cycle 1 (mean=9) and Cycle 2 (mean=6) (p=0.002). The dose tolerated significantly decreases between these two Cycles. There is also a significant decrease in dose tolerated between Cycle 1 (mean=9) and both Cycle 3 (mean =6) (p=0.000) and Cycle 4 (mean=4) (p=.004). Lastly there is a significant decrease in dose tolerated between Cycle 2 (mean=6) and Cycle 4 (mean=4) (p=0.033) (See Figure 4 and Table 3).

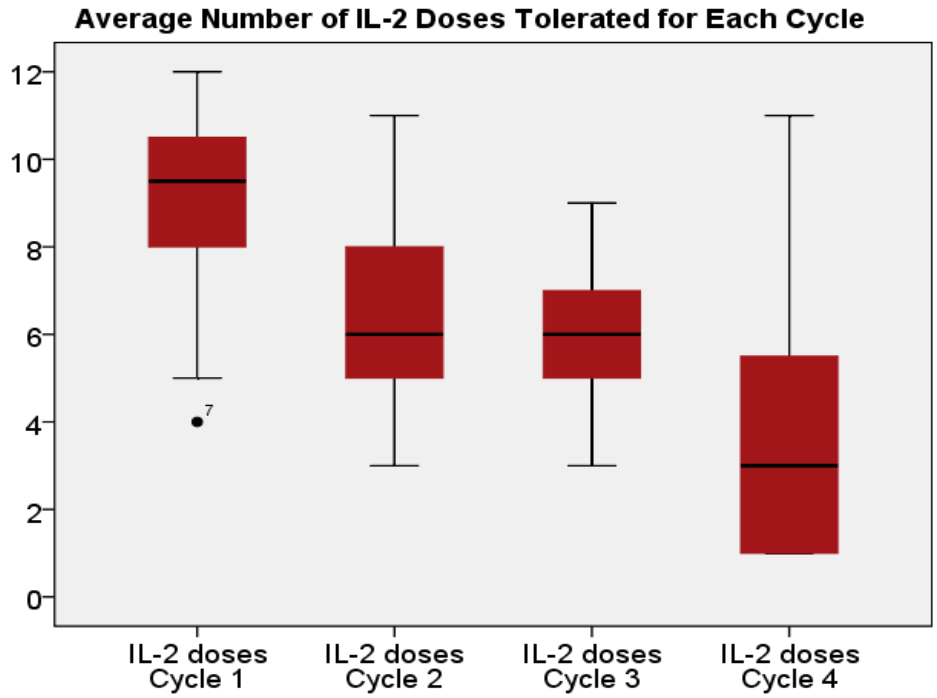


**Figure 4** Average doses tolerated by the patients for each Cycle. Highest dose tolerance occurs at Cycle 1, lowest at Cycle 4. Cycle 1 shows an outlier, patient 7, having tolerated only 4 doses.

**Table 3** Differences between the average doses tolerated by the patients for each Cycle. Determined using the paired-t test. Greatest one-step change is between Cycles 1 and 2.

| Doses Tolerated Cycle Pairs | Mean Difference | Std. Deviation | p-value |
|---|---|---|---|
| Cycle 1 – Cycle 2 | 2.529 | 2.809 | 0.002* |
| Cycle 1 – Cycle 3 | 4.091 | 2.548 | <0.001* |
| Cycle 1 – Cycle 4 | 6.286 | 3.773 | 0.005* |
| Cycle 2 – Cycle 3 | 1.182 | 2.892 | 0.205 |
| Cycle 2 – Cycle 4 | 3.857 | 3.716 | 0.033* |
| Cycle 3 – Cycle 4 | 2.143 | 4.337 | 0.239 |

*Paired samples t-test used to determine p-values
**Indicates significance

*Symptom Complexes: Mood Symptoms*

Descriptive analysis of the depressive symptom complex showed depressive symptoms peaking at Day 2 (mean=0.3375) in Cycle 1 and reaching an overall peak

value (mean=0.7045) at Day 3 in Cycle 3. Based on the abnormal distribution of values described by the Shapiro-Wilks normality test, the non-parametric Friedman test was performed. The Friedman test showed that the change in depressive symptom scores throughout Cycle 1 were not significant, p=0.735, but the changes in depressive symptom scores throughout Cycle 3 were significant, p=0.004. Due to the significance seen in Cycle 3, the non-parametric Wilcoxon test was performed. Significant differences in depressive scores were found between Cycle 3 Day 1 (mean=0.2045) and Days 2 and 3 (mean=0.6136, p=0.017 and mean=0.7045, p=0.010, respectively) (See Figure

Descriptive analysis of the anxious symptom scores showed anxious symptoms peak at Cycle 1 Day 1 (mean=0.7820), and decrease throughout the Cycle. In Cycle 3, anxious symptoms peak at Day 1 (mean=0.4843) and decrease through the Cycle. Based on the skewed distribution of values described by the Shapiro-Wilks test, the non-parametric Friedman test was performed. The Friedman test showed that the changes in anxious symptom scores throughout Cycle 1 were significant, p=0.015, but the changes in anxious symptom scores throughout Cycle 3 were not significant, p=0.391. Due to the significance seen in Cycle 1, the non-parametric Wilcoxon test was performed. Based on this test, significant differences in the depressive scores at Cycle 1 were found between Day 1 (mean=0.7820) and Day 2 (mean=0.4164) (p=0.014) and between Day 1 and Day 3 (mean=0.3820) (p=0.010). Although both depressive and anxious symptom scores show outliers, due to the small patient population, the outliers were not excluded from the analysis (See Figures 5, 6 and Table 4).

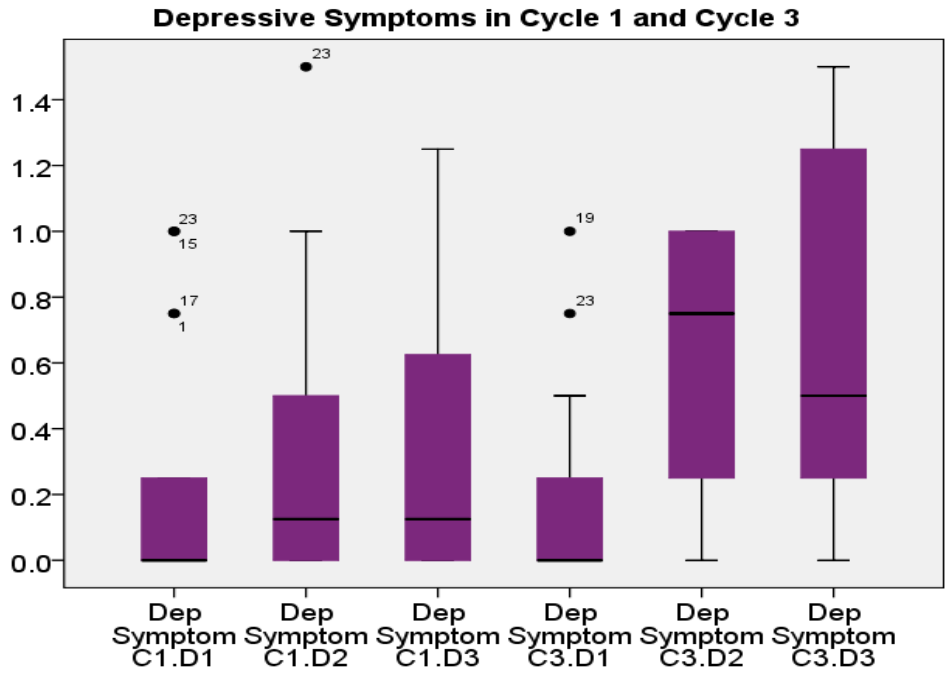


**Figure 5** Boxplot of the depressive symptom scores for Cycles 1 and 3. Depressive symptoms peak at Cycle 3 Day 3.

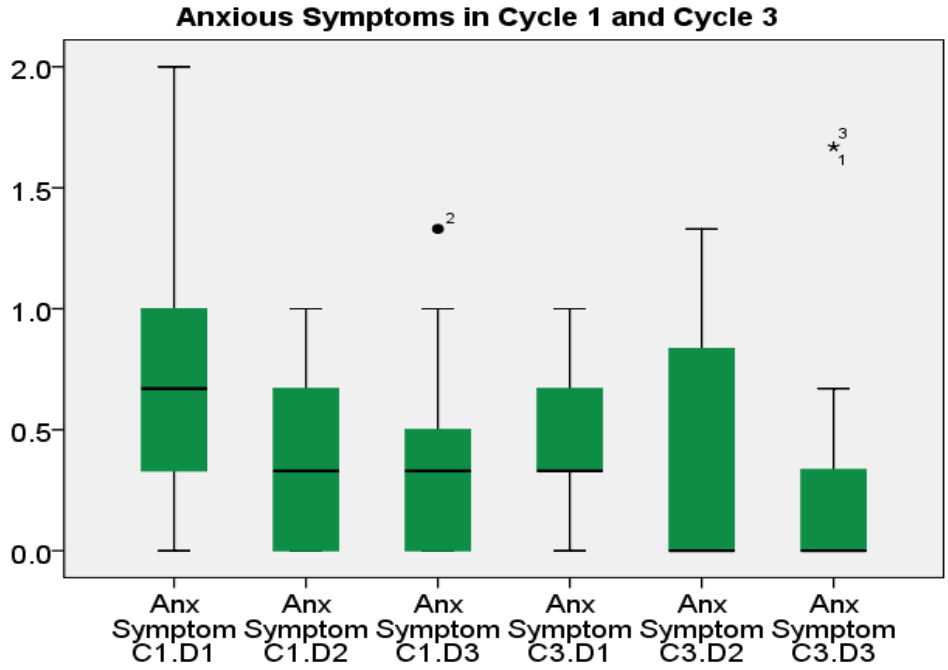


**Figure 6** Boxplot of anxious symptom scores for Cycles 1 and 3. Anxious symptoms peak at Cycle 1 Day 1.

**Table 4** Change between depressive and anxious symptoms. Significant change in depressive symptoms occurs during Cycle 3; significant change in anxious symptoms occurs during Cycle 1

| Mood Symptom Pairs | Mean Difference | Std. Deviation | p-value* |
|---|---|---|---|
| DepC3.D1 – DepC3.D2 | -.409 | .407 | 0.017** |
| DepC3.D1 – DepC3.D3 | -.500 | .387 | 0.010** |
| DepC3.D2 – DepC3.D3 | -.091 | .358 | 0.429 |
| AnxC1.D1 – AnxC1.D2 | .366 | .583 | 0.014** |
| AnxC1.D1 – AnxC1.D3 | .400 | .610 | 0.010** |
| AnxC1.D2 – AnxC1.D3 | .034 | .541 | 0.546 |

*Wilcoxon rank test used to determine p-values
**Indicates significance

*Symptom Complexes: Neurovegetative Symptoms*

The preliminary assessment of the somatic symptoms showed a peak score in Cycle 1 at Day 1 with a decreasing trend throughout the Cycle. Cycle 3 shows the opposite trend with Cycle 3 Day one starting with the lowest score and increasing throughout the Cycle to a peak of 1.182 Based on the abnormal distribution of values described by the Shapiro-Wilks test, the non-parametric Friedman test was performed. The Friedman test showed that the changes in somatic symptom scores throughout Cycle 1 were not significant, p=0.368, but the changes in somatic symptom scores throughout Cycle 3 were significant, p=0.042. Due to the significance seen in Cycle 3, the non-parametric Wilcoxon test was performed. Based on this test, significant differences in the somatic scores were found only between Cycle 3 Day 1 and Cycle 3 Day 3 (p=0.027).

The preliminary assessment of the vegetative symptom scores shows an overall peak score at Cycle 3 Day 3. The peak score in Cycle 1 is at Day 1 with no clear trend throughout the rest of the Cycle. Cycle 3 shows a clear increasing trend in vegetative scores throughout the Cycle. Based on the abnormal distribution of values described by the Shapiro-Wilks test, the non-parametric Friedman test was performed. The Friedman

test showed that the changes in vegetative symptom scores throughout both Cycle 1 and Cycle 3 were not significant, (p=0.368 and p=0.066). Again, although both somatic and vegetative symptom scores show outliers, due to the small patient population, the outliers were not excluded from the analysis (See Figures 7, 8 and Table 5).

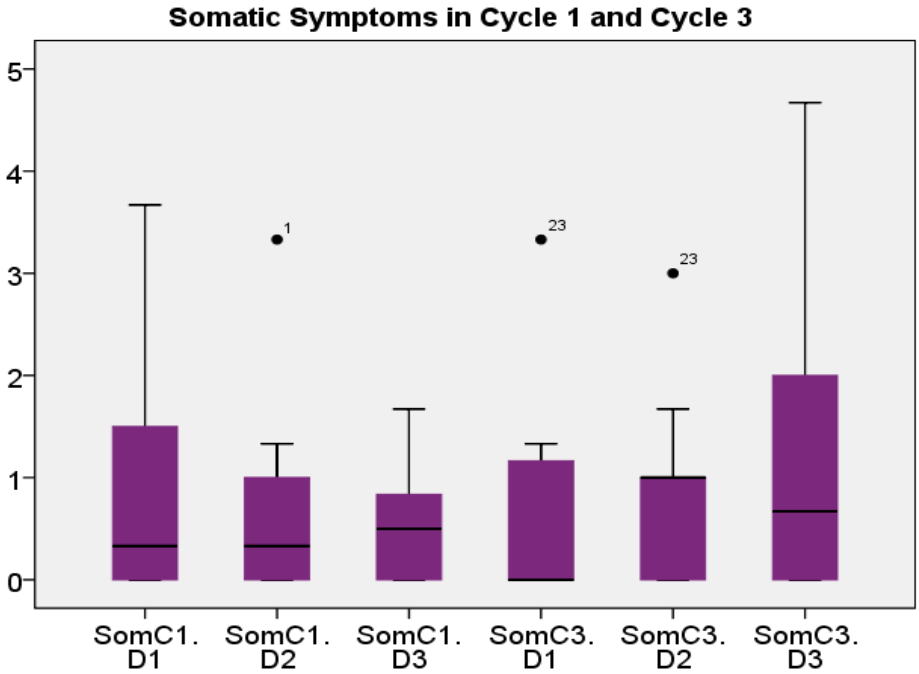


**Figure 7** Boxplot of somatic symptom scores for Cycles 1 and 3. Somatic symptoms peak at Cycle 3 Day 3.

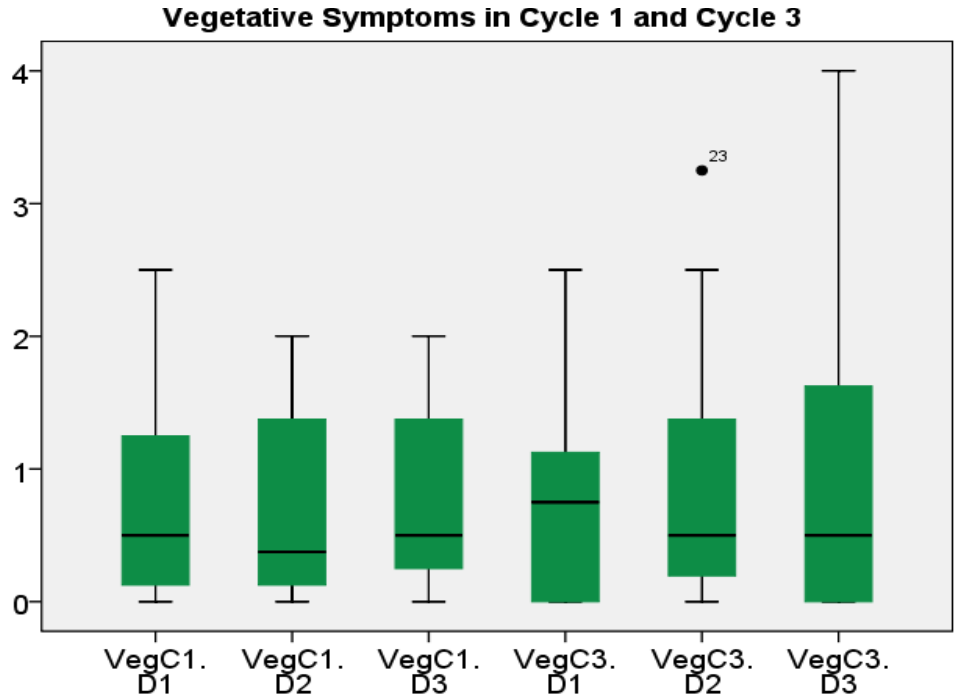


**Figure 8** Boxplot of vegetative symptom scores for Cycles 1 and 3. Vegetative symptoms peak at Cycle 3 Day 3.

**Table 5** Change between somatic symptoms in Cycle 3. Significant change in somatic symptoms occurs only during Cycle 3.

| Cycle 3 Pairs | Mean Difference | Std. Deviation | p-value* |
|---|---|---|---|
| SomC3.D1 – SomC3.D2 | -.15273 | .40430 | 0.168 |
| SomC3.D1 – SomC3.D3 | -.51636 | .60484 | 0.027** |
| SomC3.D2 – SomC3.D3 | -.36364 | .65735 | 0.104 |

*Wilcoxon rank test used to determine p-values
**Indicates significance

*CRH Receptor Expression*

The Shapiro-Wilks normality test showed CRH receptor expression to be normally distributed. Thus the paired t-test showed was used and showed no significant change in both CRHR1 and CRHR2 gene expression pre and post treatment. Also, in the preliminary analysis, it is clear that the range of expression in CRHR1 and CRHR2 values is greater in the pre-treatment population than in the post-treatment population (See Table 6, 7).

**Table 6** Descriptives of CRHR1 and CRHR2 gene expression both pre and post treatment

| Gene | Min | Max | Mean | Std Deviation |
|---|---|---|---|---|
| CRHR1 pre | 8.55 | 16.10 | 12.055 | 2.116 |
| CRHR1 post | 8.80 | 12.85 | 10.503 | 1.282 |
| CRHR2 pre | 7.27 | 15.85 | 12.174 | 2.719 |
| CRHR2 post | 11.93 | 15.23 | 13.664 | 1.280 |

**Table 7** Difference between CRHR1 and CRHR2 pre-treatment gene expression and CRHR1 and CRHR2 post-treatment gene expression. No significant difference is shown.

| Gene Expression Pairs | Mean Difference | Std. Deviation | p-value* |
|---|---|---|---|
| CRHR1 Pre-Treatment – CRHR1 Post-Treatment | 1.030 | 2.443 | 0.272 |
| CRHR2 Pre-Treatment – CRHR2 Post-Treatment | -1.075 | 2.935 | 0.370 |

*Paired sample t-test used to determine p-values
**Indicates significance

*CRHR1 Pre-treatment Expression, Mood Symptoms, and IL-2 Doses Tolerated*

For the linear regression analysis, the timepoint in Cycle 1 and Cycle 3 separately with the highest symptom complex scores was used in the analysis. Due to the Shapiro-Wilks normality test, each symptom complex score used was adjusted by adding 0.5 (to account for any zeros) and by raising it to Log 10. This was done so that an accurate parametric linear regression could be performed.

First analyzed was the question does the gene expression predict the depressive and anxious symptom scores and IL-2 doses tolerated (Gene expression= depressive + anxious + doses tolerated). The time point used in Cycle 1 for depressive scores was Cycle 1 day 2 and for anxious scores was Cycle 1 day 1. For Cycle 1, it was found that only 6.6% of the variation in gene expression is accounted for by symptom scores and IL-2 doses tolerated. Looking at each of the three variables separately, doses tolerated ($p=0.810$), depressive ($p=0.729$), anxious ($p=0.382$), none showed a p-value of significance in relation to gene expression.

The time-point used for the linear regression in Cycle 3 for depressive scores was day 3 and for anxious scores day 1. For Cycle 3 it was found that 56.8% of the variation in gene expression is accounted for by symptom scores, and IL-2 doses tolerated. Again, looking at each of the variables separately, both depressive ($p=0.167$) and anxious ($p=0.298$) symptom scores showed no significant relationship to gene expression. However, number of IL-2 doses tolerated ($p=0.030$) did show a significant relationship to CRHR1 gene expression. So, although the model as a whole is not significant, the relationship between CRHR1 gene expression and IL-2 doses tolerated in Cycle 3 is significant within this model (See Table 8).

**Table 8** Linear regression between CRHR1 gene expression and Number of IL-2 doses tolerated, depressive symptom scores, and anxious symptom scores for Cycles 1 and 3.

| Variables | C1: CRHR1 Expression | C3: CRHR1 Expression |
|---|---|---|
| **R square** | 0.066 | 0.568 |
| **B** | | |
| ***Number of IL-2 doses*** | -.063 | .891 |
| ***Depressive*** | -.905 | -3.440 |
| ***Anxious*** | 2.500 | -4.114 |
| **Std. Error** | | |
| ***Number of IL-2 doses*** | .256 | .329 |
| ***Depressive*** | 2.568 | 2.231 |
| ***Anxious*** | 2.781 | 3.659 |
| **p-value*** | | |
| ***Number of IL-2 doses*** | .810 | .030** |
| ***Depressive*** | .729 | .167 |
| ***Anxious*** | .382 | .298 |
| **ANOVA F** | 0.38 | 3.066 |
| **ANOVA P-Value** | 0.769 | .101 |

*Simple linear regression used to determine p-values
**Indicates significance

Next analyzed was the question does the variation in IL-2 doses tolerated in Cycles 1 and 3 predict both pre-treatment CRHR1 gene expression and depressive and anxious symptom scores (IL-2 doses tolerated= CRHR1 gene expression + anxious +depressive). The same time points were used for both Cycles 1 and 3. For Cycle 1, 16% of variation in IL-2 doses tolerated can be accounted for by both gene expression and depressive and anxious symptom scores. All three variables for Cycle 1, CRHR1 expression (p=0.810), depressive (p=0.358) and anxious (p=0.243) show no significant relationship with IL-2 doses tolerated.

For Cycle 3, 62.2% of variation in IL-2 doses tolerated can be accounted for by both CRHR1 gene expression and the depressive and anxious symptom scores. Similar to the last model, both depressive (p=0.284) and anxious (0.102) symptom scores show no

significant relationship with doses tolerated. However, CRHR1 expression does show a significant positive partial correlation (r=0.716, p=0.030) with IL-2 doses tolerated during Cycle 3. Upon seeing this partial correlation, we ran a Pearson correlation between Cycle 3 IL-2 doses tolerated and CRHR1 pre-treatment expression. This showed no significant correlation between the two variables (r=0.560, p=0.073) indicating that the mood symptom scores must be involved in the statistical analysis in order to see a significant relationship. Again, although the model as a whole does not show significance, the relationship between doses tolerated in Cycle 3 and CRHR1 gene expression, accounting for mood symptoms, within the model does show significance (See Table 9).

**Table 9** Linear regression between Number of IL-2 doses tolerated and CRHR1 gene expression, depressive symptom scores, and anxious symptom scores for Cycles 1 and 3.

| Variables | C1: Doses Tolerated | C3: Doses Tolerated |
|---|---|---|
| **R square** | 0.16 | 0.622 |
| **B** | | |
| ***CRHR1 expression*** | -.059 | .575 |
| ***Depressive*** | -2.311 | 2.203 |
| ***Anxious*** | -3.221 | 4.892 |
| **Std. Error** | | |
| ***CRHR1 expression*** | .243 | .212 |
| ***Depressive*** | 2.444 | 1.899 |
| ***Anxious*** | 2.657 | 2.604 |
| **p-value*** | | |
| ***CRHR1 expression*** | .810 | .030** |
| ***Depressive*** | .358 | .284 |
| ***Anxious*** | .243 | .102 |
| **ANOVA F** | 1.020 | 3.835 |
| **ANOVA P-Value** | .410 | .065 |

*Simple linear regression used to determine p-values
**Indicates significance

*CRHR2 Pre-treatment Expression, Neurovegetative Symptoms and Doses Tolerated*

Similar to the CRHR1 analysis, first analyzed was the question does the CRHR2 pre-treatment gene expression predict the somatic and vegetative symptom scores and IL-2 doses tolerated (Gene expression= somatic + vegetative + doses tolerated). The time point used in Cycle 1 for somatic scores was Day 1 and for vegetative scores, also Day 1. For Cycle 1, it was found that only 4.5% of the variation in CRHR2 gene expression is accounted for by symptom scores and IL-2 doses tolerated. Looking at each of the three variables separately, doses tolerated (p=0.799), somatic (p=0.684), vegetative (p=0.746), none showed a p-value of significance in relation to gene expression.

The time-points used in Cycle 3 for the somatic score was day 3 and for the vegetative score was day 3. For Cycle 3, the regression showed that 21.9% of the variation in gene expression is accounted for by symptom scores and IL-2 doses tolerated. Again, looking at each of the variables separately, doses tolerated (p=0.685) depressive (p=0.167) and anxious (p=0.298) symptom scores showed no significant relationship to gene expression. Due to the statistically significant result of Pearson's correlation between somatic and vegetative symptom scores (p=0.011) the linear regression model was run using somatic and vegetative symptom scores separately; however no significant change was seen in the model using this method; somatic (p=0.473) and vegetative (p=0.502) (See Table 10).

**Table 10** Linear regression between CRHR2 gene expression and Number of IL-2 doses tolerated, somatic symptom scores, and vegetative symptom scores for Cycles 1 and 3.

| Variables | C1: CRHR2 Expression | C3: CRHR2 Expression |
|---|---|---|
| **R square** | 0.045 | 0.219 |
| **B** | | |
| ***Number of IL-2 doses*** | -.080 | .314 |
| ***Somatic*** | 1.075 | -3.062 |
| ***Vegetative*** | .992 | -1.623 |
| **Std. Error** | | |
| ***Number of IL-2 doses*** | .308 | .743 |
| ***Somatic*** | 2.595 | 5.684 |
| ***Vegetative*** | 3.011 | 6.615 |
| **p-value*** | | |
| ***Number of IL-2 doses*** | .799 | .685 |
| ***Somatic*** | .684 | .607 |
| ***Vegetative*** | .746 | .813 |
| **ANOVA F** | .250 | 0.656 |
| **ANOVA P-Value** | .860 | 0.604 |

*Simple linear regression used to determine p-values
**Indicates significance

Again, similar to CRHR1 analysis, the next analysis was the question does the variation in IL-2 doses tolerated in Cycles 1 and 3 predict both pre-treatment CRHR2 gene expression and somatic and vegetative symptom scores (IL-2 doses tolerated= CRHR2 gene expression + somatic + vegetative). The same time points were used for both Cycles 1 and 3. For Cycle 1, 2.2% of variation in IL-2 doses tolerated can be accounted for by both gene expression and somatic and vegetative symptom scores. All three variables for Cycle 1, CRHR2 expression (p=0.799), somatic (p=0.698) and vegetative (p=0.983) show no significant relationship with IL-2 doses tolerated.

For Cycle 3, 46.2% of variation in IL-2 doses tolerated can be accounted for by both CRHR2 gene expression and the somatic and vegetative symptom scores. Similar to

the last model, CRHR2 expression (p=0.685), somatic (p=0.698) and vegetative (0.150) symptom scores show no significant relationship with doses tolerated (See Table 11).

**Table 11** Linear regression between Number of IL-2 doses tolerated and CRHR2 gene expression, somatic symptom scores, and vegetative symptom scores for Cycles 1 and 3.

| Variables | C1: Doses tolerated | C3: Doses tolerated |
|---|---|---|
| **R square** | 0.022 | 0.462 |
| **B** | | |
| ***CRHR2 expression*** | -.052 | .079 |
| ***Somatic*** | -.830 | -1.165 |
| ***Vegetative*** | -.053 | 4.601 |
| **Std. Error** | | |
| ***CRHR2 expression*** | .202 | .188 |
| ***Somatic*** | 2.100 | 2.881 |
| ***Vegetative*** | 2.444 | 2.850 |
| **p-value*** | | |
| ***CRHR2 expression*** | .799 | .685 |
| ***Somatic*** | .698 | .698 |
| ***Vegetative*** | .983 | .150 |
| **ANOVA F** | .121 | 2.007 |
| **ANOVA P-Value** | .947 | .202 |

*Simple linear regression used to determine p-values
**Indicates significance

*ACTH, IL-6 and Gene expression*

Based on the Shapiro-Wilks normality test, the non-parametric Spearman's correlation was performed on the time points in Cycle 1 and Cycle 3 with the highest levels of ACTH and IL-6 and on the pre-treatment CRHR1 and CRHR2 gene expression. The only significant correlation seen is between CRHR1 expression and levels of IL6 on Cycle 1 Day 2 (p=0.051) (See Table 12).

**Table 12** Correlation between CRHR1 and CRHR2 pre-treatment gene expression and the highest levels of both ACTH and IL-6 in Cycles 1 and 3. Significant correlation is found only between IL-6 and CRHR1 gene expression at Cycle 1 Day 1.

| | | ACTH Cycle 1 Day 1 | ACTH Cycle 3 Day 1 | IL6 Cycle 1 Day 2 | IL6 Cycle 3 Day 2 |
|---|---|---|---|---|---|
| **CRHR1** | *Correlation Coefficient* | -.132 | -.539 | -.480 | -.018 |
| | *Sig. (2-tailed)** | .613 | .108 | 0.051** | .960 |
| **CRHR2** | *Correlation Coefficient* | .071 | -.370 | -.076 | -.042 |
| | *Sig. (2-tailed)* | .786 | .293 | .772 | .907 |

*Spearman's correlation used to determine p-values
**Indicates significance

## Discussion

As a whole, our study sample consists of a fairly homogenous group of patients, thus there was no need to control for demographic characteristics. However, our patients are skewed towards one end of the spectrum making our findings less globally applicable. Our patients were 75% male thus eliminating the need for any gender-specific analyses. This is most likely due to the exclusion of patients who have depressive symptoms and/or are on antidepressants at the screening visit as evidenced by the HAM-D and HAM-A screening scores. This exclusion, however, may have eliminated a higher proportion of females than males from our study due to the higher incidence rates of depression in females (Massie 2004). Our patient population is also 90% Caucasian, again limiting the global applicability of the study. This, however, is most likely due to the higher incidence rates of melanoma in Caucasians than other ethnic groups (Cress 1997).

In regards to IL-2 doses tolerated and patient attrition data, it is clear that dose tolerance decreases significantly per cycle, perhaps due to induction of adverse side effects. Also note that the highest attrition rates are following Cycle 2. This is most likely due to the patient reevaluation to determine whether they are fit to continue IL-2

treatment following Cycle 2. If a patient's cancer has progressed the patient was discontinued from the IL-2 treatment as well as the study. Thus, after Cycle 2 potentially those affected most severely by the IL-2 are withdrawn from the study. This then biases our results towards the null hypothesis.

**Prediction** *IL-2 doses tolerated will decrease by each cycle, with cycle 1 having the highest average doses tolerated and cycle 4 having the least average doses tolerated.*

In support of our prediction, patients are able to tolerate the most doses of IL-2 in Cycle 1 and the least number of doses in Cycle 4. In general, most patients undergoing IL-2 therapy are able to handle 8-12 doses in the first cycle of treatment. The general average number of doses then decreases with each cycle. Our patients show an average dose tolerance of 9 in Cycle 1, falling in the lower end of the average range. Our patients' average dose tolerance also decreases throughout the cycles. However, it is not a linear decrease in tolerance that is shown. The most significant decrease in tolerance occurs between Cycle 1 and Cycle 2. There is no change between cycles 2 and 3 and another decrease, though not as severe as between cycles 1 and 2, between cycles 3 and 4. The negligible change between cycles 2 and 3 is most probably explained by the longer break between cycles and, again, the patient reevaluation. As those patients who are most negatively affected by the IL-2 treatment leave the study, the most resilient patients are left increasing the total average amount of doses tolerated.

**Prediction** *Each symptom complex average will gradually increase within both Cycle 1 and Cycle 3 with an overall peak occurring at Cycle 3 Day 3.*

In support of our prediction, depressive symptoms did increase throughout cycle 1 and did increase significantly throughout cycle 3. The depressive symptom peak is cycle

3 day 3. The most significant increase in depressive scores is between cycle 3 day 1 and cycle 3 day 2, however the change between cycle 3 day 2 and cycle 3 day 3 is insignificant. This could perhaps be explained by both an increasing resistance to the neuropsychological effects of IL-2 treatment within the cycle and to the decreasing dose tolerance within the cycle.

Although depressive symptom data supported our prediction, the anxious symptom data showed an opposite trend, decreasing significantly in cycle 1 and decreasing insignificantly in cycle 3. This could potentially be explained by the fact that patients are usually more anxious and nervous before beginning a cycle of IL-2 treatment. The patients seem to be especially nervous/anxious on the first day of treatment, thus explaining the overall anxious peak at cycle 1 day 1. As treatment continues, however, either the physical side effects induced by the treatment overshadow or subdue their anxiety, or they become more accepting of the treatment. Patients are also nervous after cycle 2. Upon passing the patient reevaluation and entering cycle 3, patients become more aware of the fact that the next two cycles of treatment are their last opportunity of getting the cancer under control as there are few non-clinical trial treatment options available after IL-2. Although we did not investigate cycle 4 within this study, our findings would lead us to predict that we may see an opposite trend in cycle 4 as the patients' final cycle of treatment comes to an end.

However, it should be noted that a very plausible explanation for the opposite trend seen in the anxious symptom scores could be from the fact that patients are allowed to be on anxiolytics upon beginning the study, and are frequently prescribed benzodiazepines throughout treatment. Thus, if a patient is experiencing high levels of

anxiety on the first day of Cycle 1, that patient may be prescribed a benzodiazipine for the rest of the cycle, thereby dampening our measurements.

In regards to neurovegetative symptoms, both vegetative and somatic symptom data shows no visible trend in cycle 1. However, vegetative symptoms insignificantly increase throughout cycle 3 and, as predicted, have an overall peak at cycle 3 day 3, and somatic symptoms significantly increase throughout cycle 3 and also have an overall peak at cycle 3 day 3. The only significant change in somatic symptoms occurred between cycle 3 day 1 and cycle 3 day 3 indicating a gradual yet still significant development in these symptoms throughout treatment.

**Prediction** *IL-2 treatment will have no significant effect on both CRHR1 and CRHR2 expression, thus there will be no significant difference between the pre-treatment expression and the post-treatment expression.*

In agreement with our prediction, no significant change was seen between pre and post IL-2 treatment gene expression (both CRHR1 and CRHR2). This indicates that IL-2 treatment does not effect the expression of these receptors, receptors that have been postulated as having a role in the morphology of depression. However, it should be noted that because this was a clinical study, translation of CRHR1 and CRHR2 could not be measured only plasma samples, instead of pituitary tissue where CRHR1 and CRHR2 are found (Schwartzentruber 2001), could be taken thus adding some doubt into this finding.

An interesting trend between the pre and post treatment gene expression is that the range of delta Ct values decreases between pre and post treatment. We believe this is due to the fact that those most resilient to the negative effects of IL-2 treatment are the ones that make it through the entire treatment, and therefore have a post-treatment

assessment. Because there was no significant changes in post treatment expression in either gene, only that the delta Ct values were more clustered, perhaps having either too high or too low CRHR1 and CRHR2 expression has more to do with increased susceptibility to the negative neuropsychological side effects of IL-2 treatment than only having too high expression. However, this prediction needs to be further investigated using a larger patient sample.

*CRHR1 Regression Models*

**Prediction** *Variability in CRHR1 pre-treatment gene expression will be accounted for by the depressive and anxious symptom scores and IL-2 doses tolerated in both Cycle 1 and Cycle 3.*

Only 6.6% of the variation in gene expression could be accounted for by depressive and anxious symptom scores and IL-2 doses tolerated in cycle 1, thereby not supporting our prediction. However, in cycle 3 the symptom scores and doses tolerated account for a vastly increased 56.8% of *CRHR1* gene variation though the overall model is still not statistically significant.

**Prediction** *We predict that variability in IL-2 doses tolerated in Cycle 1 and Cycle 3 will be accounted for by the depressive and anxious symptom scores in Cycles 1 and 3 as well as CRHR1 pre-treatment gene expression.*

The *CRHR1* gene expression and symptom scores only account for 16% of the variation in IL-2 doses tolerated in Cycle 1, also not supporting our prediction. Again, however, in Cycle 3 the symptom scores and gene expression account for an increased 62.2% of variation in tolerance.

Although both CRHR1 regression models are not significant and do not support the entirety of the original prediction, it does support our idea that patients will show an increased neuropsychiatric response to treatment in Cycle 3 as opposed to Cycle 1. This is perhaps due to their biological familiarity with the treatment resulting in an increased and faster immune response, thus inducing the HPA axis pathway discussed previously.

Again, although the entire models were not significant, we did see a significant positive partial correlation between CRHR1 expression and IL-2 doses tolerated during Cycle 3 in both CRHR1 regression models. This indicates that variation in CRHR1 expression does, in fact, appear to be related to a patient's ability to tolerate IL-2 while accounting for the differences in mood symptoms. Although it is a positive correlation, the opposite of what was expected, it should be noted that by Cycle 3 the number of patients has decreased to 11, thus, the post-treatment expression showed that variability in the CRHR1 expression is decreasing as the patient population becomes more resilient to the treatment. This could potentially explain the reverse trend that is seen as patients may be withdrawing from the study for reasons other than depressive and anxious symptoms. This suspected relationship may be further elucidated as more patients participate in the study, which may allow the overall model to become significant.

*CRHR2 Regression Models*

**Prediction** *Variability in CRHR2 pre-treatment gene expression will be accounted for by the somatic and vegetative symptom scores and IL-2 doses tolerated in both Cycle 1 and Cycle 3.*

For cycle 1, only 4.5% of the variation in gene expression could be accounted for by somatic and vegetative symptom scores and IL-2 doses tolerated in Cycle 1. Although

we saw a significant increase in R-squared scores in the previous two CRHR1 regression models between Cycle 1 and Cycle 3, the increase seen in this CRHR2 regression model between Cycles 1 and 3 is not as high. In Cycle 3 the somatic and vegetative symptom scores and doses tolerated account for only 21.9% of gene variation.

**Prediction** *Variability in IL-2 doses tolerated in Cycle 1 and Cycle 3 will be accounted for by the somatic and vegetative symptom scores in Cycles 1 and 3 as well as CRHR2 pre-treatment gene expression.*

For the second CRHR2 regression run, similar to the first, the gene expression and somatic and vegetative symptom scores account for merely 2.2% of the variation in gene expression, thereby not supporting our prediction. However, the symptom scores and gene expression account for an increased 46.6% of variation in tolerance in Cycle 3. Despite the statistical insignificance of both CRHR2 regression models, it is interesting to note the difference in R-square scores between Cycles 1 and 3 in the second neurovegetative model is more than double that of the first model.

**Prediction** *Expression of both CRHR1 and CRHR2 pre-treatment will correlate with levels of ACTH and IL-6, supporting the HPA axis model described earlier.*

The pre-treatment expression of both CRHR1 and CRHR2 in the plasma had insignificant correlations with the highest level of ACTH during cycles 1 and 3. However, CRHR1 pre-treatment expression did correlate with IL-6 in cycle 1. This indicates a potential relationship between CRH receptor expression, or more generally receptors involved in HPA axis signaling and the pro-inflammatory immune response. On another note, we did find a pattern in peak levels of IL-6 and ACTH each cycle indicating a cyclic relationship between the two, and perhaps a delay in the effects of IL-2

treatment. The peak levels of ACTH occur during Day 1 of every cycle while the peak levels of IL-6 occur during Day 2 of every cycle.

Our study did have some significant biases which should be taken into consideration upon evaluation of our research. Perhaps one of the greatest confounders in this study is the limitation that our larger, double-blind parent study places on us. We are unable to take into account the effect pre-treatment with escitalopram may have had on our patients. Escitalopram has been shown to alter the HPA axis pathway and induce the upregulation of glucocorticoid receptors, though no previous studies have shown its effects on CRHR1 and CRHR2 receptor expression (Uys et al. 2005). Thus, based on this evidence, escitalopram may also affect, either directly or indirectly, CRHR1 and CRHR2 post treatment gene expression. The reason for the smaller range in CRHR1 and CRHR2 expression post-IL-2 treatment then may be because the vast majority of patients who made it through the treatment were in the escitalopram treatment group.

Another limitation is the study exclusion criteria; patients who are depressed or on anti-depressants are ineligible for the study. As IL-2 treatment is the last possible treatment for these stage IV melanoma patients, patients eligible for IL-2 have most likely been through a variety of cancer treatment such as radiation therapy, chemotherapy, surgery, and IFN-a immunotherapy. As stated previously, it has been shown that IFN- $\alpha$ induces depressive symptoms in 45% of patients treated with the immunotherapeutic drug. Thus, if patients had gone through IFN- $\alpha$ treatment, experienced the depressive side effects, and were prescribed anti-depressants they are ineligible for this study upon beginning IL-2 therapy. This presumably gives us a patient

population consisting of the most resilient patients. A larger, more comprehensive, observational study looking at a broader range of patients should solve this bias.

Also interesting, though not included in this analysis of this paper, our lab has previously found that cortisol levels within these IL-2 patients remain low and exhibit no significant changes throughout treatment. Rat studies have shown that CRHR1-/- mutants are unable to regulate cortisol secretion. Future research might examine the relationship between cortisol and CRHR1 and CRHR2 pre-treatment gene expression (Schwartzentruber 2001). Perhaps a way to do this is to examine CRHR1 and CRHR2 gene expression in relation to the results of the dexamethasone suppression test before and after IL-2 treatment.

The next logical step might be to determine the amount of CRH in the blood plasma of patients along with IL-1 and TNF- $\alpha$ levels in order to complete the HPA axis/immune system pathway. For the larger study, analysis of the first pathway, the tryptophan catabolism pathway, discussed in the background needs to be analyzed and how these two pathways interact and impact the outward symptoms of the patient will complete the analysis. A larger sample size would increase the power of the study and thereby allow significance of study outcomes. The future unblinding of study treatment groups will allow use to evaluate the efficacy of the treatment escitalopram on the patients' well-being and quality of life.

Further research on the relationship between CRHR1 and CRHR2 expression and patients' tolerance to IL-2 treatment along with the biological and behavioral effect pre-treatment with an SSRI has on these patients may help increase the patients' ability to withstand more IL-2 doses throughout treatment and overall increase their quality of life.

Clinically, the subsequent increase in IL-2 dose tolerance will allow for an increased probability of cancer regression and an extended life for these patients.

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