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Jennifer Koch Date

Relationship between Inflammation and Diurnal Cortisol Secretion in Patients with Major Depression: Role of Tumor Necrosis Factor Alpha

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Relationship between Inflammation and Diurnal Cortisol Secretion in Patients with Major Depression: Role of Tumor Necrosis Factor Alpha

By

Jennifer Koch
B.S. James Madison University, 2012

Advisor: Andrew Miller, M.D.

An abstract of
A thesis submitted to the Faculty of the
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Abstract

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Major Depressive Disorder (MDD) has been shown to be associated with high inflammation, including high levels of inflammatory cytokines such as tumor necrosis factor (TNF), as well as glucocorticoid resistance and disrupted diurnal cortisol secretion. It is believed that cortisol feedback inhibition is suppressed by inflammatory cytokines such as TNF. A double blind, clinical trial was conducted in which 60 patients, who met the criteria for MDD, were enrolled and either administered a placebo or the TNF antagonist infliximab over a period of 8 weeks. Blood samples were collected at weeks 1 and 8 and these samples were analyzed via ELISA assays for cortisol and TNF levels and were used to determine gene expression. This study determined that the administration of infliximab was able to restore natural diurnal cortisol rhythms and that TNF antagonists may be useful in lessening depressive symptoms.

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Introduction:

Depression is associated with inflammation

Recent data suggest that activation of inflammatory pathways may play a role in behavioral disturbances including depression. For example, the presence of increased plasma and cerebrospinal fluid (CSF) concentrations of inflammatory cytokines and markers of immune activation such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6, tumor necrosis factor alpha (TNF-alpha), acute phase reactants, chemokines, and cellular adhesion molecules has been noted in depression (Maes et al., 1991; Raison et al., 2006). Meta-analyses of this literature suggest that the cytokines IL-6 and TNF and the acute phase reactant c-reactive protein (CRP) may be the most reliably elevated in depressed patients versus controls (Howren et al., 2009; Dowlati et al., 2010; Zorrilla et al., 2001). Of note, not all depressed patients exhibit increased inflammatory markers, such that depression is not an inflammatory disorder per se, and increased inflammation has been found in other neuropsychiatric disorders including bipolar disorder, schizophrenia and anxiety disorders (Raison et al., 2011). Another reason inflammation is believed to play a role in depression is that inflammatory cytokines have been shown to interact with nearly every pathophysiologic pathway relevant to depression such as neurotransmitter metabolism, neuroendocrine function, synaptic plasticity, and regional brain activity (Dantzer et al., 2008; Mueller N and Schwarz M, 2007; Wichers et al, 2005; Miller et al., 2009). In addition, administration of inflammatory cytokines such as IFN-alpha, IL-1, IL-6, and TNF-alpha have been shown to lead to “sickness behavior” in laboratory animals and humans, in which many symptoms of depression such as alterations in mood, neurovegetative function, and

cognition are present (Dantzer et al., 2004). Indeed, administration of IFN-alpha to patients suffering from cancer or hepatitis C results in many of the key diagnostic criteria of major depression such as depressed mood, irritability/anxiety, anhedonia, impaired sleep, decreased appetite, psychomotor retardation, fatigue, and cognitive dysfunction (Raison et al., 2006).

These studies further support the theory that inflammation may play a role in depression and is evidence of lessened depression severity upon treatment with anti-inflammatory medications. For instance, in a large double-blind placebo-controlled trial, patients were administered the TNF-alpha antagonist, etanercept, in order to treat their psoriasis (Tyring et al., 2006). Patients who received etanercept displayed significantly greater decreases in depressive symptoms as compared to controls (Tyring et al., 2006). Additionally administration of acetylsalicyclic acid, an inhibitor of cyclooxygenase-1 and 2 and the production of prostaglandins, increased remission rates in patients with major depression, previously unresponsive to fluoxetine, when combined with fluoxetine therapy (Mendlewicz et al., 2006). Furthermore, depressed patients who received celecoxib, a selective cyclooxygenase-2 inhibitor, in combination with reboxetine displayed greater antidepressant effects than those administered reboxetine alone (Mueller et al., 2006). Finally, in a 12 week study in which patients with major depression were administered infliximab, a TNF-alpha antagonist, those with high CRP concentrations at baseline (>5 mg/L), showed lessening of a wide range of depressive symptoms, including depressed mood, psychomotor retardation, and performance of work and other activities (anhedonia/fatigue), as well as psychic anxiety and suicidal ideation upon treatment with infliximab compared to placebo (Raison et al., 2013). A

wealth of literature therefore supports the theory that inflammation plays a role in depression, leading to speculation on the potential mechanisms by which proinflammatory cytokines and other immune molecules contribute to depression and what alterations that characterize depression may predispose depressed patients to inflammation.

Glucocorticoids and Inflammation

One of the primary homeostatic regulators of the inflammatory response is glucocorticoids. Endogenous glucocorticoids regulate the immune response by inhibiting the production and release of various cytokines and inflammatory mediators and coordinating immune cell distribution throughout the body (Evans et al., 2005; Raison et al., 2006). In the clinic, synthetic glucocorticoids are some of the most common agents used to treat autoimmune and inflammatory disorders (Quan et al., 1999).

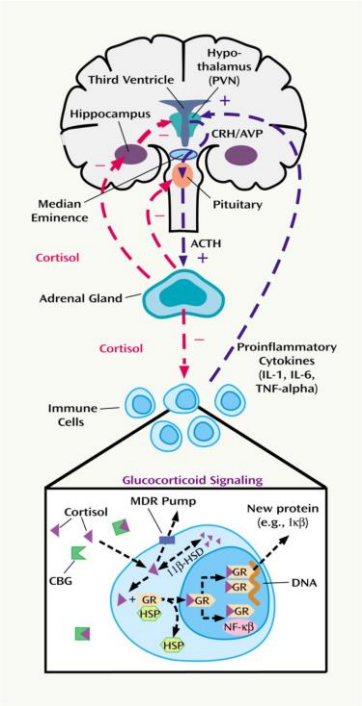

Figure 1. Schematic representation of the HPA axis (Raison CL and Miller AH, 2003).

Endogenous glucocorticoids such as cortisol are released from the hypothalamic-pituitary-adrenal (HPA) axis in response to stress and as a result of the diurnal cycle (Yirmiya et al., 1996). Specifically, corticotropin-releasing hormone (CRH) is released by the hypothalamus from the median eminence where it enters the portal circulation to induce the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which then enters the bloodstream and acts within the cortex of the adrenal glands to release cortisol in humans (Owens and Nemeroff,

1991) (**Figure 1**). Cortisol is then able to act on the hippocampus, pituitary, and hypothalamus to regulate its own secretion, creating a negative regulatory feedback loop (Yirmiya et al., 1996; Raison et al., 2006) (**Figure 1**).

Inhibition of inflammatory signaling by glucocorticoids occurs via protein-protein interactions between the glucocorticoid receptor (GR) and the inflammatory transcription factor nuclear factor kappa B (NF-kB) as well as activator protein-1 (AP-1) (De Bosscher et al, 2014). Binding of glucocorticoids to GRs prevents proinflammatory transcription factors such as NF-kB and AP-1 from similarly binding these receptors and therefore represses an inflammatory response from occurring in the cytosol (De Bosscher et al, 2014). In this way glucocorticoids such as cortisol are able to regulate inflammatory responses.

Inflammation/Cytokines can disrupt Glucocorticoid Signaling

Interestingly, inflammatory cytokines have been shown to be able to disrupt GR signaling (Miller et al., 1999), and thereby potentially disrupt glucocorticoid regulation of the immune response as well as the HPA axis. Studies have shown that cytokines can interact with GR signaling pathways and thereby disrupt glucocorticoid action (Miller et al., 1999). Indeed cytokines have been shown to decrease GR translocation from cytoplasm to nucleus, decrease GR DNA binding

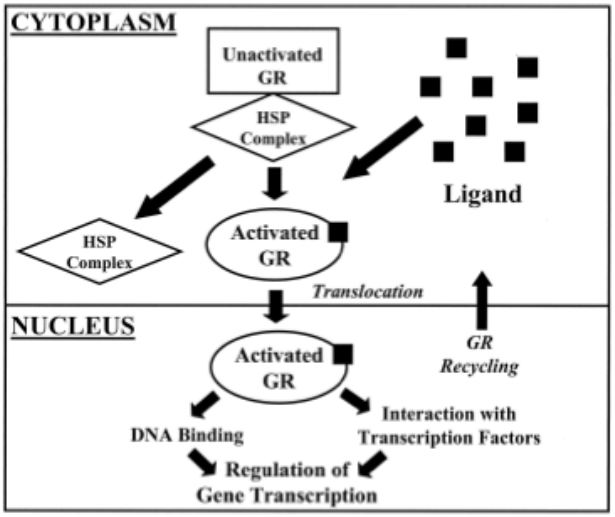

Figure 2. "Nucleocytoplasmic traffic" model of glucocorticoid receptor (GR) activation. (Pariante and Miller, 2001)

and decrease activation of relevant GR-inducible enzymes or reporter gene constructs (Miller et al., 1999; Miller et al., 2001; Wang et al., 2004). Patients with chronic inflammatory diseases such as sepsis, asthma, ulcerative colitis, acquired immunodeficiency syndrome, rheumatoid arthritis, and allogenic organ transplant have also shown changes in GR number and function such as reduced effects of glucocorticoids on functional endpoints (Corrigan et al., 1991; Spahn et al., 1996; Weidenfeld et al, 1996; Leung et al., 1995), reduced GR affinity for ligands (Shimada et al., 1993; Sher et al., 1994; Norbiato et al., 1996), and reduced GR upregulation (Shimada et al., 1993; Sher et al., 1994; Norbiato et al., 1996; Krasznai et al., 1986).

GRs when "unactivated" are localized to the cytoplasm where they are associated with a multimeric complex of chaperon proteins including several heat shock proteins (HSPs) (Pratt et al., 1993) (**Figure 2**). The GR is bound by steroid in the cytoplasm and then undergoes a conformational change through which it is activated, causing it to dissociate from the chaperon protein complex and translocate to the nucleus (Guiochon-Mantel et al., 1996). In the nucleus, the GR either binds to hormone response elements (HREs) on DNA or interacts with other transcription factors (Guiochon-Mantel et al., 1996) (**Figure 2**).

As an example of the effects of cytokines on the GR, the proinflammatory cytokine IL-1alpha has specifically been shown to be able to disrupt GR signaling. IL-1alpha is thought to inhibit translocation of the GR from the cytosol to the nucleus, as evidenced by increased concentrations of GR protein and increased GR binding and immunostaining in the cytoplasm as well as decreased translocation from cytoplasm to nucleus following in vitro administration of the synthetic glucocorticoid dexamethasone

(Pariante et al., 1999; Wang et al., 2004). It is believed that p38 MAPK pathways are involved in IL-1alpha-mediated GR disruption as IL-1alpha effects on GR function were attenuated upon administration of a selective p38 MAPK inhibitor and were completely reversed by antisense oligonucleotides targeted to p38 (Wang et al., 2004). Proinflammatory cytokines like IL-1alpha bind to their membrane receptors and activate many MAPK pathways besides p38 including the ERK, JNK, and NF-kB pathways as well as activating other inflammatory molecules like signal transducer and activator of transcription 5 (STAT5) (Stylianou et al., 1998; Pace et al., 2007). These pathways are believed to also disrupt nuclear protein-protein interactions which inhibit GR-DNA binding (Pace et al., 2007). Cytokines such as IL-1 produced during an inflammatory response may affect the capacity of glucocorticoids to transmit signals to target tissues and induce GR resistance in certain cell types, contributing to immune influence on the HPA axis and disease expression (Pariante et al., 1999).

Psychosocial stress has also been shown to affect inflammation and GR function. For example, peripheral blood mononuclear cells from individuals exposed to chronic caregiver stress were found to exhibit increased expression of genes regulated by NF-kB, whereas gene expression regulated by the GR was found to be decreased (Miller et al., 2008). Psychosocial stress in laboratory animals has also been shown to result in increased inflammation in association with decreased GR translocation and function, an effect that was eliminated in IL-1 receptor knockout mice (Engler et al., 2008).

Role of Glucocorticoid Receptors in Regulating Cortisol Secretion-Negative Feedback

As noted above**,** a role of GRs is to regulate the secretion of glucocorticoids, cortisol being the primary glucocorticoid in humans. Depression is characterized by HPA axis hyperactivity and impaired HPA axis glucocorticoid feedback sensitivity (Pariante and Miller, 2001). Some patients with major depression in fact have been shown to exhibit increased cortisol concentrations in plasma, urine, and cerebrospinal fluid (CSF) as part of an exaggerated response to ACTH and an enlargement of the pituitary and adrenal glands (Gold et al., 1988; Holsboer and Barden, 1996; Nemeroff et al., 1996; Owens and Nemeroff, 1993). This HPA hyperactivity is believed to be due to the failure of cortisol to suppress CRH production through negative feedback regulation; a phenomena known as

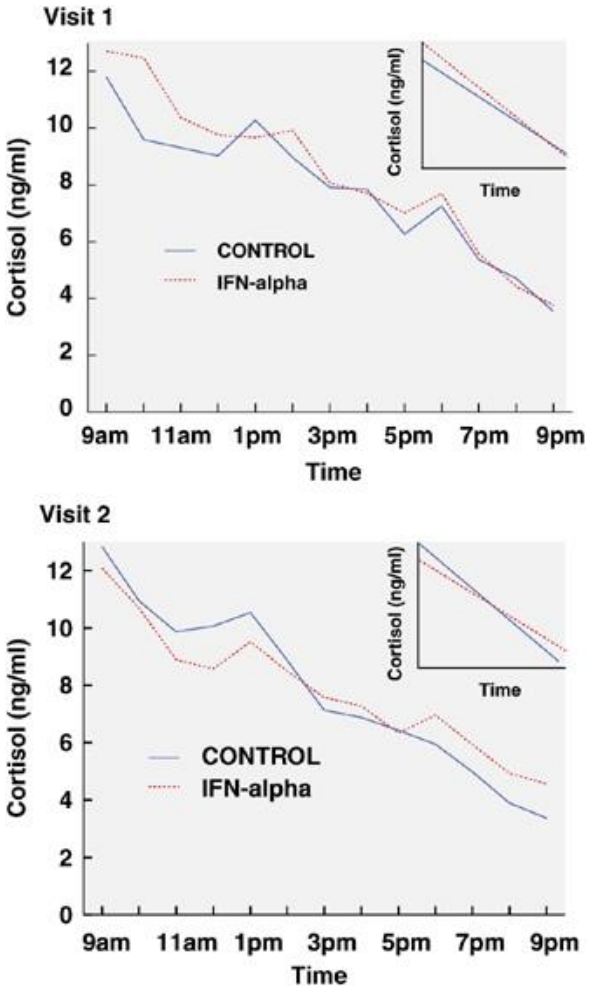

Figure 3. Mean raw cortisol values from 0900 to 2100 hours in controls (blue line) versus subjects treated with interferon plus ribavirin (red line) at visit 1 and 2. Compared to control subjects, IFN-α/ribavirin-treated patients exhibited a significantly flatter cortisol slope ($P<0.05$) and significantly higher evening cortisol values ($P<0.05$).

glucocorticoid resistance (Holsboer et al., 2000; Pariante and Miller, 2001). Glucocorticoid resistance is evidenced by the fact that cortisol non-suppression occurs in response to dexamethasone administration in the dexamethasone suppression test (DST). The DST determines GR sensitivity because dexamethasone should reduce ACTH release and therefore cortisol levels in normal subjects (Holsboer et al., 2000; Pariante and Miller, 2001). Rates of impaired glucocorticoid responsiveness measured via DST (as evidenced by high cortisol concentrations >5ng/dl the morning following evening 11 pm dexamethasone administration) have been found to vary between 25-80% in depressed patients (Holsboer et al., 2000). Thus although glucocorticoids are elevated in patients with depression, inflammatory responses may not be inhibited due to insufficient GR signaling (Raison and Miller, 2003).

Impact of Cytokines on Cortisol Rhythm

Cortisol secretion occurs in a diurnal cycle with cortisol levels at their highest point in the morning (Edwards et al., 2001). Throughout the rest of the day, this cortisol peak declines with lowest levels of cortisol secretion occurring in the early evening hours (Edwards et al., 2001). Inflammatory cytokines have been shown to disrupt normal daily patterns of cortisol and flatten the cortisol curve by increasing evening levels of cortisol (Raison and Miller, 2003; Matthews et al., 2006; Ticher et al., 1996; Rosmond and Bjorntorp, 2000). For instance, administration of IFN-alpha to patients with hepatitis C infection led to significant alterations in diurnal HPA axis activity with an increase in evening corticol and flattening of the diurnal curve (**Figure 3**)(Raison et al., 2010). Of note, flattening of the cortisol curve has been associated with a variety of medical illnesses and predicts reduced survival in cancer patients (Abercrombie et al., 2004;

Sephton et al., 2000). Disruption in diurnal cortisol rhythm is also associated with depression (Deuschle et al., 1997; Raison et al., 2010). In fact, the flattening of the cortisol curve has been associated with glucocorticoid resistance as evidenced by non-suppression of glucocorticoid secretion following dexamethasone administration (Spiegel et al., 2006). Thus inflammatory cytokines through disruption of GR function may lead to increased evening cortisol levels and flattening of the cortisol curve (**Figure 3**). As demonstrated by Raison et al., subjects treated with IFN-alpha exhibited a significantly flatter cortisol slope ($p<0.05$) and significantly higher evening cortisol values ($p<0.05$) than non-IFN-alpha-treated control subjects (Raison et al., 2010) (**Figure 3**). Nevertheless, no studies have examined the relationship between inflammatory cytokines and diurnal cortisol secretion in depressed subjects, and no studies have explored whether this relationship is cause and effect. In order to elucidate this relationship, we measured plasma concentrations of TNF and cortisol before and after administration of the TNF antagonist infliximab and determined whether TNF-related changes in cortisol secretion were reversed by TNF blockade. We hypothesized that antagonism of inflammatory cytokines would restore normal diurnal cortisol patterns in patients with depression and high inflammation.

Methods:

Study Procedures

A randomized, double-blind trial was conducted in which individuals diagnosed with major depression or bipolar disorder depressed and treatment resistance according to a score of ≥2 on the Massachusetts General Hospital Staging (MGH-S) scale (a clinician administered Antidepressant Treatment response questionnaire, used to determine treatment resistance) were administered either the TNF-alpha antagonist infliximab or a placebo (Raison et al., 2013). Sixty subjects both male and female aged 25-60 years who were on a consistent antidepressant regimen or off antidepressant therapy for at least 4 weeks prior to baseline were enrolled. Antidepressant regimens were required to remain stable throughout the study and could include conventional antidepressant medications, mood stabilizers, antipsychotic medication, stimulants and benzodiazepines. Exclusion criteria included any autoimmune disorder (confirmed by laboratory testing); history of tuberculosis (confirmed by chest X-ray, skin and blood testing) or high risk of tuberculosis exposure; hepatitis B or C or human immunodeficiency virus infection (confirmed by laboratory testing); evidence of active fungal infection; history of recurrent viral or bacterial infections; history of cancer excluding basal cell or squamous cell carcinoma of the skin (fully excised with no recurrence); unstable cardiovascular, endocrinologic, hematologic, hepatic, renal, or neurologic disease (determined by physical examination and laboratory testing); history of schizophrenia (determined by Structured Clinical Interview for DSM-IV - SCID)(First et al., 1997); active psychotic symptoms of any type; substance abuse/dependence within the past 6 months (determined by SCID); active suicidal ideation determined by a score ≥3 on item #3 on the17-item

Hamilton Depression Rating Scale (HAM-D-17)(Hamilton 1960) and/or a score <28 on the Mini-Mental State Exam (Folstein et al., 1975). All subjects provided written informed consent to the study, and all study procedures were approved by the Emory University Institutional Review Board. The study was registered in ClinicalTrials.gov, Identifier: NCT00463580.

Clinical Assessments

Depression diagnosis was established according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Depression severity was assessed, using the Hamilton Depression Rating Scale (HAM-D-17) at baseline and after 8 weeks of treatment. A response to treatment was considered a 50% reduction in HAM-D-17 score from baseline at any point during the 12 week study.

Infliximab and Placebo Infusion Procedures

Subjects were administered 5mg/kg of infliximab or placebo through an indwelling catheter at the Emory Division of Digestive Diseases at baseline, 2 weeks, and 6 weeks. Infliximab is a chimeric monoclonal antibody grown in mammalian cells that is unable to cross the blood brain barrier that is used to antagonize TNF-alpha (**Figure 4**). It is FDA-approved for the treatment of diseases such as hepatitis C, psoriasis, Crohn's

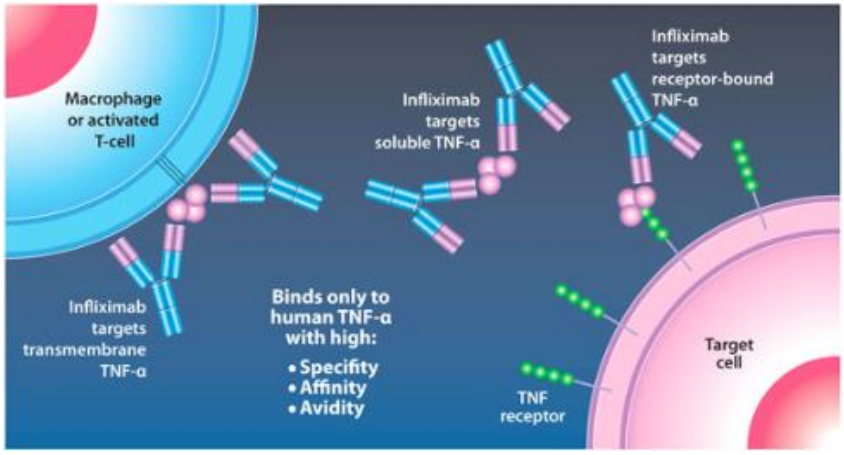

Figure 4. Infliximab structure and mechanism of action. http://www.peterbullartstudio.co.uk/webcontent/?cat=8

disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, and ulcerative colitis. The standard intravenous induction routine used for the treatment of inflammatory bowel disease was employed in this study. This protocol entailed 3 infusions given over 6 weeks (baseline, 2 weeks and 6 weeks). Infliximab or placebo was dispensed by independent pharmacists in a 250ml saline bag according to a computer-generated randomization list, blocked in units of 4 and provided by a study statistician. The saline-dissolved placebo was matched to infliximab in color and consistency. Study staff remained blinded to group assignment throughout the duration of the trial. When signs of infection occurred, infusions were delayed until symptoms resolved and/or appropriate treatment was administered. Medications for hypertension, diabetes, hypothyroidism, allergies, infections and other medical conditions as well as low doses of aspirin (81mg per day) were allowed as dictated by the patients' treating physicians. The infliximab and placebo solutions were provided, free of charge, by Centocor Ortho Biotech Services (Horsham, PA).

Biological Samples

Whole blood was collected from patients at 9:00, 10:00, 11:00, 12:00, 13:00, 14:00, 15:00, 16:00, 17:00, 18:00, 19:00, 20:00, and 21:00 time points at baseline and 24 hours after the first infusion and again at 8 weeks (2 weeks after the week 6 infusion). Plasma was extracted from whole blood and stored at -80C until cytokine levels were determined via ELISA kits (IBL International and R&D Systems) according to manufacturer instructions. ELISA stands for enzyme-linked immunosorbent assay and is able to measure cortisol and other inflammatory cytokine concentrations in blood plasma via the conjugation of these proteins to specific antigens plated on various different 96

well plates, an enzymatic substrate is then added to produce a reaction and a detectable signal, in the case of the aforementioned assays: color change. Color change was detected using a spectrophotometer, and the color intensity of plasma samples was compared to a set of standards to determine cytokine concentration at 450 and 650 nm wavelengths. Cortisol levels were evaluated at all time points (IBL International, Toronto, ON). TNF-alpha levels were measured using early morning samples (9:00/10:00) (R&D Systems, Minneapolis, MN). Concentrations of TNF-alpha and cortisol were determined via fluorescence intensity on the SpectraMax 340PC (Molecular Devices, Sunnyvale, CA) and transformed using GraphPad Prism 6 Software.

Whole Blood was also collected for gene expression analysis in Tempus Blood RNA Tubes (Applied Biosystems, Carlsbad, CA) at baseline (9 am). Tempus tubes were stored at -80C until RNA was extracted via RNeasy kits (QIAGEN, Valencia, CA) according to manufacturer instructions (**Figure 5**). RNA sample concentrations and purity were determined using the MBA 2000 System (Perkin-Elmer, Shelton, CT, USA). Each sample was linearly amplified by WT-Ovation RNA amplification system (NuGEN) and then submitted to the Emory Cancer Genomics

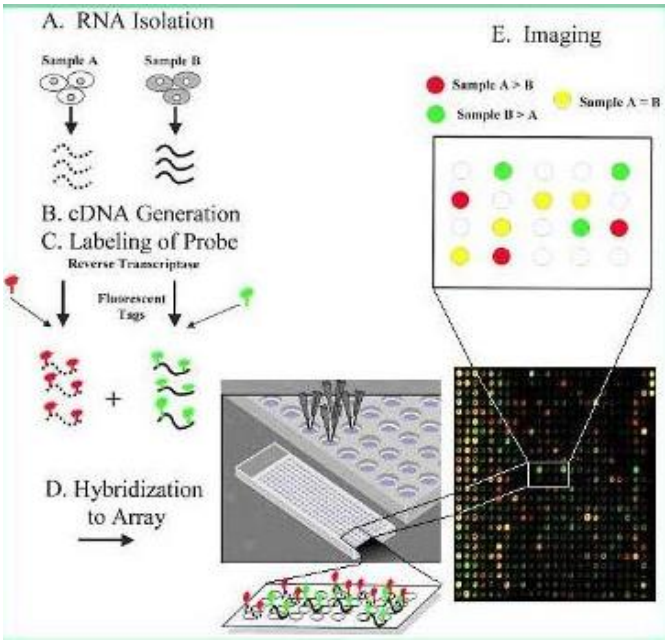

Figure 5. Diagram of sample preparation and microarray analysis. http://www.eufic.org/article/de/ernahrung/nutrigenome/expid/review-nutrition-genome/

Core for microarray analysis, where RNA was reverse transcribed into cDNA and a labeling probe was attached (**Figure 5**). After hybridization to Illumina Human HT-12 Expression BeadChips (Illumina, San Diego, CA), BeadChips were scanned on the Illumina BeadArray Reader to determine raw probe fluorescence intensity (**Figure 5**).

Statistical Analysis

The raw OD values determined from the cortisol assays via spectrophotometer were converted into cortisol concentrations (ng/ml) using GraphPad Prism software. These cortisol measurements were then statistically analyzed using IBM SPSS software. Of the 60 subjects included in the trial, 54 subjects had complete neuroendocrine and immune data and were included in the analyses. Descriptive statistics were used to characterize the dependent variables. T tests and Chi Square (or Fisher's Exact) tests were used to compare means or percentages between relevant groups of interest. For cortisol assessments morning (AM cortisol) maximums, evening (PM cortisol) minimums and the difference between AM and PM (AM/PM cortisol) were determined in order to investigate diurnal hormonal variations. As previously described, morning peak value (AM cortisol) was chosen as the highest cortisol value between the blood draws at 0900 and 1100 hours (Raison et al., 2010). Evening minimum (PM cortisol) was similarly calculated at the lowest cortisol value of the last blood draws at 1900 and 2100 hours (Rasion et al., 2010).

The raw probe fluorescence intensity of the gene expression BeadChips determined by the Illumina BeadArray Reader was exported from the Illumina Genome Studio software into the R program (http://www.R-project.org) for statistical analysis. The data was normalized and ln transformed using *vsn* (Huber et al., 2002) and corrected

for hybridization batch effects using an Empirical Bayes estimation (Johnson et al., 2007). The association between gene expression in patients determined to have high versus low PM cortisol were compared using generalized linear models in R, while adjusting for the effects of age, gender, BMI and medication status. Gene transcripts differentially expressed in patients with high PM cortisol levels as compared to those with low PM cortisol levels were defined as having a ≥20% difference (1.2 fold change) corresponding to a <10% FDR (Cole et al., 2003; Miller et al., 2008; Torres et al., 2013) and cut-off of $p \leq 0.01$ (Haider et al., 2008), and were therefore highly predictive based on both statistical significance (p value) as well as biological effect size (fold change). Predictions were performed using nearest shrunken centroids (NSC) method (*pamr*) with a leave out one 100-fold cross validation scheme to ensure robustness of the prediction (Tibshirani et al., 2002). Functional annotation of transcripts within pathways was assessed using MetaCore software (GeneGo Inc., St. Joseph, MI). Differentially expressed genes were also analyzed using a network-based transcription factor analysis in MetaCore (Ekins et al., 2007).

Results:

Demographic and Clinical Variables

Demographic and clinical variables did not differ significantly between the infliximab-treated and control groups in the sample as a whole (n=60) (Raison et al., 2013) or in the 54 patients included in the present study. Distributions and comparisons of the demographic and clinical variables for both the infliximab and placebo-treated groups are shown in **Table 1**. No differences in gender, ethnicity, age, BMI, baseline HAM-D-17 depression scores, Week 8 HAM-D-17 depression scores, or the number of individuals on current antidepressants, mood stabilizers, antipsychotic medicines and psychotropic medicines were observed between the groups (all $p>0.05$).

Baseline Relationships between Neuroendocrine and Immune Parameters

Linear regression analyses were used to identify significant predictors of baseline cortisol parameters including AM cortisol, PM cortisol, and AM/PM cortisol values. Preliminary linear regression analysis found that sex was a significant predictor of BL PM cortisol values ($t=2.14$, $p=0.038$) therefore subsequent analysis were conducted in males and females separately. Sex has also been previously been shown to significantly influence inflammatory status (Eisenberger et al., 2009), therefore the dataset was split by sex and the relationship between females and males and cortisol parameters were analyzed seperately. The relationship between BL PM cortisol and BL TNFalpha was significantly stronger in females (ß =0.33, $p=0.048$) (**Figure 6**) than in males (ß =-0.05, $p=0.82$) (**Figure 7**). The male patients were then removed from further analysis. As some patients were treated with the TNFalpha antagonist infliximab and some patients were treated with a placebo, treatment was applied to the regression analysis as a between

subjects factor with age, BMI, psychotropic meds being controlled for yielding a significant relationship between BL PM cortisol and BL TNFalpha (p=0.049, F=4.538).

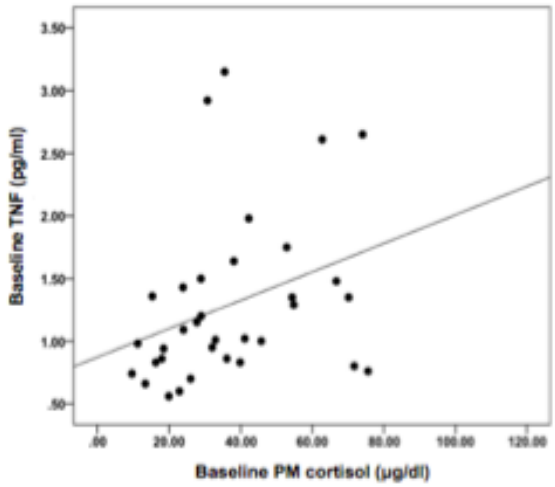

Figure 6: T-Test of BL PM Cortisol levels and BL TNFalpha levels in females (ß=0.33, p=0.048).

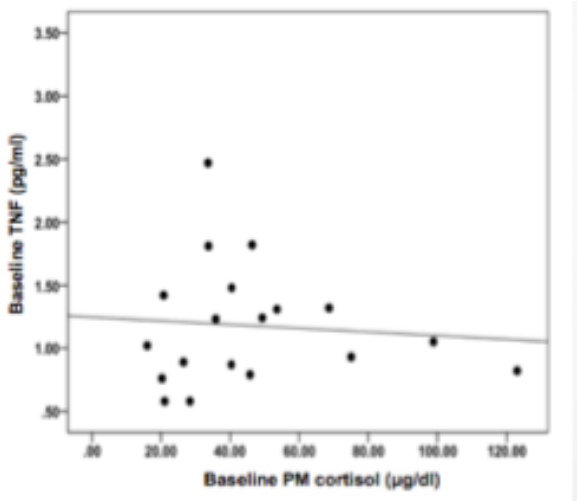

Figure 7. T-Test of BL PM cortisol levels and BL TNFalpha levels in males (ß=-0.05, p=0.82).

Effects of TNF-alpha Antagonism on PM Cortisol concentrations

As previously observed, patients with higher BL TNFalpha values were generally more responsive to infliximab (Raison et al., 2010; Mehta et al., 2013) therefore approximately the bottom quarter of patients with the lowest TNFalpha values were taken out of the analysis; yielding a stronger relationship between BL PM cortisol and BL TNFalpha. Given the relationship between increased TNF-alpha and increased PM cortisol in high TNFalpha females at baseline, repeated measure ANOVAs were used in women with increased plasma TNF-alpha (TNF-alpha > 0.85 pg/ml) to assess the relationship between baseline PM cortisol levels and 8 week PM cortisol levels as a function of treatment, time and their interaction. A significant treatment by time interaction was found ($F=5.848$, $p=0.026$) with baseline PM cortisol levels reducing in the infliximab but not the placebo treated group (**Figures 8, 9, 10**). A significant correlation was also found between TNF-alpha and AM-PM cortisol levels, baseline AM-PM cortisol levels reduced in the infliximab but not the placebo treated group ($F=4.804$, $p=0.040$)(**Figures 11, 12, 13**).

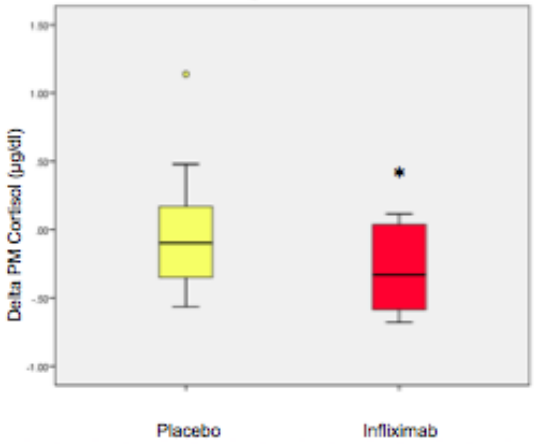

Figure 8: Infliximab treatment decreases PM cortisol levels in females. 8 week PM cortisol levels were subtracted from baseline PM cortisol levels then divided by baseline PM cortisol levels and were grouped by treatment.

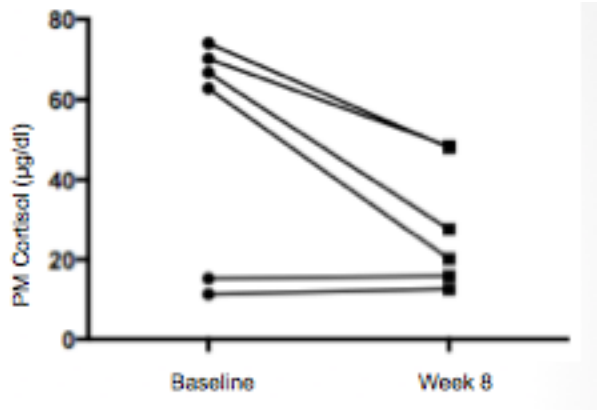

Figure 9. Infliximab treatment in PM cortisol levels in high TNFalpha females at baseline and week 8.

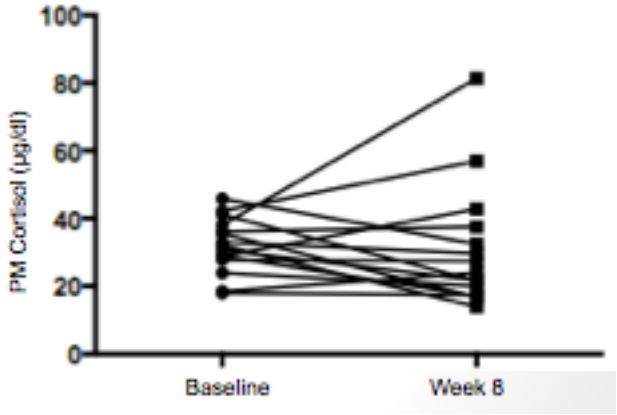

Figure 10. Placebo treatment in PM cortisol levels in high TNFalpha females at baseline and week 8.

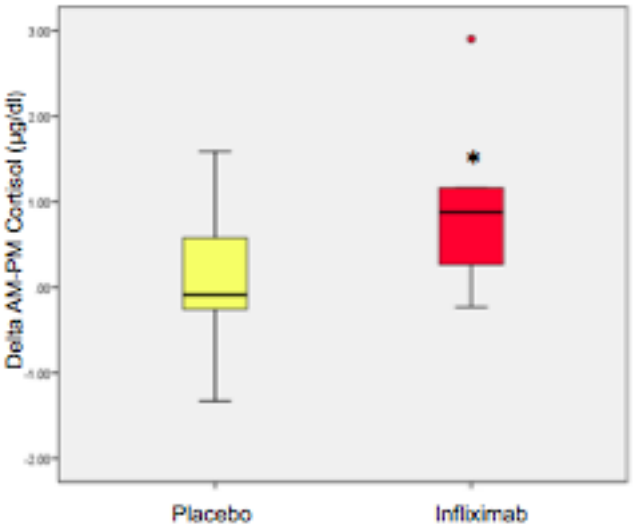

Figure 11: Infliximab treatment increases delta AM-PM cortisol values. 8 week delta AM-PM cortisol values were subtracted from baseline delta AM-PM cortisol values and were grouped by treatment.

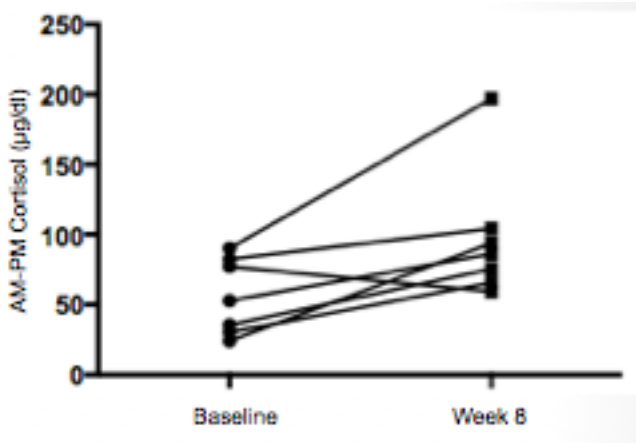

Figure 12. Infliximab treatment in AM-PM cortisol values in high TNFalpha females at baseline and week 8.

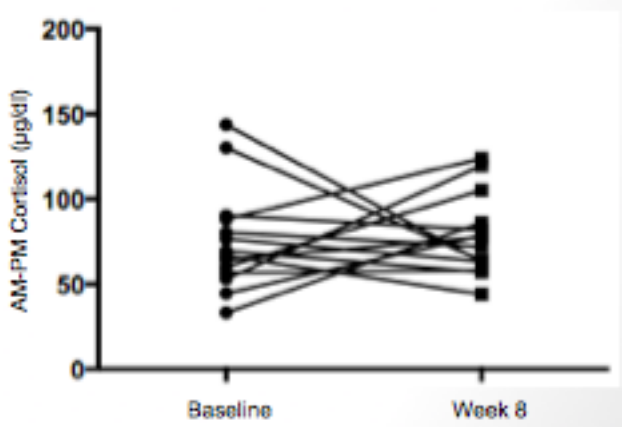

Figure 13. Placebo treatment in AM-PM cortisol values in high TNFalpha females at baseline and week 8.

Gene Expression

As previously described, baseline gene expression profiles in the 10 depressed patients with the highest PM cortisol versus the 10 patients with the lowest PM cortisol adjusting for sex, race, age, BMI and use of psychotropic medications were determined in R to be differentially expressed (1.2 fold change, adjusted $p \leq 0.01$) and then compared using Metacore. At baseline these depressed patients showed an abnormal immune response as normally stress hormones such as cortisol induce leukocyte mobilization in a process known as chemotaxis, however as seen in **Figure 14**, chemotaxis is in actuality found to be downregulated (Lewitus et al., 2008). Additionally upregulation of inflammatory cytokines such as TNFR2 may be causal in HPA axis dysfunction in the depressed patients (**Figure 14**). It will be interesting to see in future studies whether after infliximab administration chemotaxis was induced and TNFR2 downregulated, leading to normal secretion of cortisol and functioning of the HPA axis.

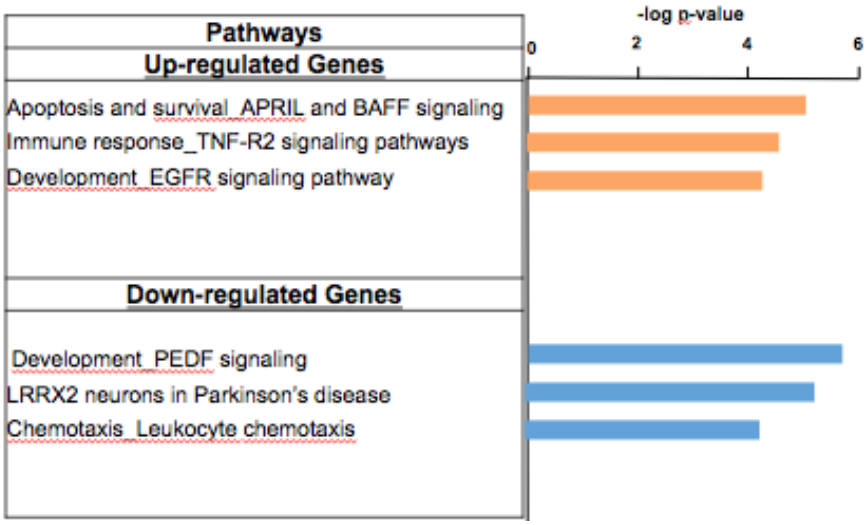

Figure 14. Upregulated and downregulated gene pathways as determined by Metacore software.

Discussion:

Summary of results

Significant correlations were found between plasma TNFalpha concentrations and plasma PM cortisol concentrations in females. Infliximab treatment was found to significantly decrease PM cortisol concentrations in females with high TNF concentrations as compared to those treated with placebo. Additionally infliximab treatment was shown to increase the difference between the AM cortisol concentrations and PM cortisol concentrations. The decrease in PM cortisol concentrations restored the PM cortisol levels similar to that of healthy non-depressed patients. This superiority between infliximab and placebo treatment was noted in patients with high baseline TNFalpha concentrations (>0.85 ng/ml). Genomic analysis further showed upregulation of TNF family genes.

Relationship between cytokines and the HPA axis

This is one of the first studies of its kind to find a link between immune variables and HPA axis alterations in patients with depression. The correlation between TNFalpha and PM cortisol levels provides a mechanism by which the HPA axis may be disrupted and may alter behavior and produce many of the symptoms of depression and other illnesses. These findings confirm previous studies such as that by Raison et al. which found that administration of IFNalpha to patients with hepatitis C increased TNFalpha and TNFR2 and implicated the increase of these factors in disruption of normal HPA axis activity (Raison et al., 2010). In addition, in previous studies IL-6 was found to be correlated with dampened cortisol rhythms in patients with colon cancer and increased

PM cortisol in patients with coronary artery disease (Rich et al., 2005; Nijm et al., 2007). Correlations have also been found between IL-6, IL-10, and sIL-2R and awakening cortisol response (Wichers et al., 2007). These data along with our own support findings that cytokines can disrupt HPA axis regulation, possibly through effects on the GR receptor.

Mechanism

It is believed previously mentioned increases in cytokines may in fact be causative in disruption of HPA axis activity including diurnal cortisol rhythm. The restoration of diurnal cortisol rhythms via blockade of TNFalpha, by means of infliximab administration, leads one to believe that TNFalpha may in fact be interfering with HPA axis activity and the regulation of these rhythms. TNFalpha and PM cortisol levels, those most affected by depression, were actually found to be significantly correlated, implying that manipulation of one may affect the other. Furthermore, the fact that gene pathways related to TNF-R2 was shown to be significantly upregulated at baseline, supports the theory that TNFalpha and its binding of its soluble receptors interferes with HPA axis diurnal activity.

There are several theories by which this mechanism may operate which further research will need to explore. Inflammatory cytokines have previously been shown to activate HPA axis pathways through corticotropin releasing hormone (Sapolsky et al., 1987; Besedovsky et al., 1996). Additionally cytokines have been found to inhibit glucocorticoid receptor function, thereby disrupting feedback regulation of the HPA axis and leading to higher PM ACTH and cortisol levels (Pace et al., 2007; Pariante et al.,

1999). Also as previously mentioned, cytokines have been shown to decrease GR translocation from cytoplasm to nucleus, decrease GR DNA binding and decrease activation of relevant GR-inducible enzymes or reporter gene constructs (Miller et al., 1999; Miller et al., 2001; Wang et al., 2004). We believe that cytokines such as TNFalpha may therefore preventing GR-DNA binding and thus preventing feedback of cortisol to the HPA axis; leading to hypersecretion of CRH and ultimately higher cortisol production. Therefore by lowering levels of cytokines like TNFalpha with drugs like infliximab, feedback regulation of the HPA axis may be restored; preventing immune mediated depression.

Relevance to other illnesses

The data reported here corroborate the findings of a number of studies which show that dysregulation of the HPA axis diurnal activity is a consequence of a variety of illnesses and may be the link between these illnesses and behavioral disturbances. For instance, flattening of the cortisol rhythm has been noted in a number of illnesses including cancer, type 2 diabetes, and cardiovascular disease, all of which are associated with inflammation (Matthews et al., 2006; Ticher et al., 1996; Rosmond et al., 2000; Abercrombie et al., 2004). Flattening of the cortisol slope in these diseases has been linked to depression, fatigue, anxiety, and maladaptive coping styles (Deuschle et al., 1997; Giese-Davis et al., 2004; Bower et al., 2005; Adam et al., 2001; Polk et al., 2005; Barnett et al., 2005). Given that many of these illnesses have been associated with increased cytokines, these data suggest that increased inflammation may be a common denominator between illness and impaired HPA axis function.

Limitations

Several limitations of the study are to be noted. First, the sample size was relatively small, therefore the significant correlations found between cortisol and TNFalpha may be secondary to a lack of power. Additionally although sex differences have been noted in previous studies in which inflammatory cytokine levels have been measured (Eisenberger et al., 2009), the effect was only observed in females and not in males; therefore whether inflammatory cytokines play a role in male depression is still unknown. The hypothesis predicts that glucocorticoids are unable to feedback to GR receptors, however GR number and function were not measured and therefore we have an incomplete picture of glucocorticoid sensitivity. Additionally a low number of cytokine parameters were assessed, therefore it is unknown whether other cytokines that were not measured could also be interfering with HPA axis function. In conducting gene expression analysis comparison between high and low cortisol was made on a relatively small sample size, which may have affected the ability to detect group differences, however a number of highly significant transcripts (n=148) were in fact detected. Additionally a full cortisol curve was not able to be determined in the time limitations of this thesis, providing less points in which to calculate AM and PM cortisol levels. I believe many of these limitations could be corrected if the study was continued and conducted on a larger scale, with glucocorticoid sensitivity and additional inflammatory cytokines being measured.

Conclusions

Infliximab did show superiority over placebo treatment as an effective means of restoring diurnal cortisol rhythms, leading one to believe that blockade of TNF is a viable

strategy for treatment of certain depressive symptoms. This difference was also particularly observed in females over males, a phenomena that has been previously been observed in other studies evaluating the effects of cytokine-induced depression (Eisenberger et al., 2009). As sex differences in both depression and inflammatory disorders are well known, with females twice as likely as males to develop depression (Nolen-Hoeksema et al., 2001) and 2-9 times more likely to develop an autoimmune disorder (Whitacre et al., 1999), we believe that the absence of a correlation between high cytokine levels and HPA axis disruption to be expected. We believe if the sample size was bigger we would also see this development in males. The correlation between TNFalpha and HPA axis activity as well as the previously discovered relationships between other cytokines and HPA axis function gives further validity to the theory that increased cytokine levels may play an important role in the pathology of various diseases. In particular these findings provide strong evidence for the treatment of disorders associated with diurnal cortisol rhythm dysfunction such as major depressive disorder with a TNFalpha antagonist like infliximab. The patients in this study suffered no adverse effects associated with infliximab administration as compared with placebo, thereby implicating TNFalpha antagonists as a viable therapy option.

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Tables:

Table 1. Demographic and clinical variables in the total patient sample, patients treated with infliximab, and patients treated with placebo.

	All	Infliximab	Placebo
Gender			
Female	35 (64.8%)	15 (62.5%)	20 (66.7%)
Male	19 (35.2%)	9 (37.5%)	10 (33.3%)
Race			
Caucasian	44 (81.5%)	21 (87.5%)	23 (76.7%)
Other	10 (18.5%)	3 (12.5%)	7 (23.3%)
Age	43.6 (8.83)	41.9 (8.22)	43.3 (9.44)
BMI	31.3 (7.32)	29.4 (5.57)	32.7 (8.04)
Baseline HAM-D-17	23.8 (3.88)	24.1 (4.04)	23.6 (3.80)
Week 8 HAM-D-17	16.4 (7.21)	17.9 (8.06)	13.1 (7.93)
Current antidepressant			
Yes	31 (57.4%)	12 (50.0%)	20 (66.7%)
No	23 (42.6%)	12 (50.0%)	10 (33.3%)
Current antipsychotic			
Yes	7 (13.0%)	3 (12.5%)	4 (13.3%)
No	47 (87.0%)	21 (87.5%)	26 (86.7%)
Current mood stabilizer			
Yes	6 (11.1%)	2 (8.33%)	3 (10.0%)
No	48 (88.9%)	22 (91.7%)	27 (90.0%)
Current psychotropic			
Yes	33 (61.1%)	13 (54.2%)	21 (70.0%)
No	21 (38.9%)	11 (45.8%)	9 (30.0%)