

**THE IMPACT OF SINGLE NUCLEOTIDE POLYMORPHISMS ON  
HPA AXIS FUNCTIONALITY IN DEPRESSION**

by

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## ABSTRACT

Depression is one of the most prevalent risks to human productivity and life worldwide, decreasing life expectancies by up to 7-10 years. The hypothalamic-pituitary-adrenal (HPA) axis plays a primary role in stress response through the regulated secretion of the glucocorticoid hormone cortisol. Diseases of cortisol dysregulation such as Cushing's syndrome (hypercortisolemia) and Addison's Disease (hypocortisolemia) are both associated with depression. Based on this we, and others, have hypothesized that mutations in the genes for the glucocorticoid receptor (GR), the closely related mineralocorticoid receptor (MR), and regulatory proteins associated with cortisol or GR function may contribute to depression in the absence of hyper- or hypo-cortisolemia. Our study investigated the genotypic frequency in the clinical population of several single nucleotide polymorphisms (SNPs) that affect GR and MR sensitivity to cortisol binding. Buccal swab DNA samples were acquired from patients clinically diagnosed with depression and from a random population. Extracted DNA was analyzed utilizing multiple allele-specific polymerase chain reactions to determine genotypic frequency of SNPs associated with hypersensitivity or resistance to cortisol. In addition, patients completed measures of depression (BDI) and anxiety (STAI-T, STAI-S). While there was no significant difference between genotypes for BDI measures, patients carrying a TthIII mutant allele for the GR had significantly higher scores on the STAI-T inventory. Further understanding the role of genotypic variation in cortisol function could lead to more specific and targeted therapies for depression with the goal of improving patient outcomes.

## INTRODUCTION

Stress and the resulting neuroendocrine responses have been evolutionarily essential to survival, accessing energy reserves and instigating a myriad of adaptations in physiology and behavior that relieve the adverse stressor (DeRijk & de Kloet, 2005). In contrast, long-term prolonged stress can additionally affect function of physiological processes such as the sympathetic nervous system and immune response (Mackin & Young, 2004) and disrupt or degrade cellular development, communication and overall health. Further, abnormal functioning or impaired regulation of stress response systems and their subsequent products has been strongly linked to physiological and psychiatric disorders including Major Depressive Disorder (Dougherty, Klein, Olino, Dyson, & Rose, 2009) This is particularly true of early-developmental adversity, which predisposes one to depressive symptoms and conditions (Cai, 2015)(Brown et al., 1987).

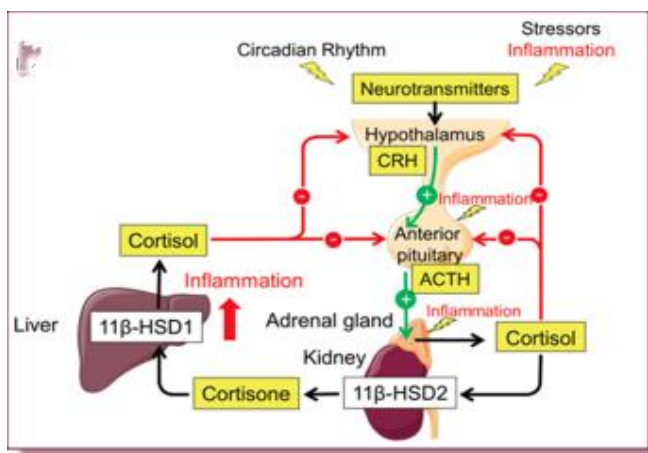
Diagnosis of depression is determined by presence of 5 of 9 depressive symptoms, with one being either depressed mood or anhedonia. These symptoms as determined by DSM-5 are:

- |                         |                       |                          |
|-------------------------|-----------------------|--------------------------|
| 1. Depressed mood;      | 5. Psychomotor        | 8. diminished ability to |
| 2. Markedly diminished  | agitation or          | think or concentrate,    |
| interest or displeasure | retardation           | or indecisiveness        |
| 3. Increase or decrease | 6. fatigue or loss of | 9. recurrent thoughts of |
| in either weight or     | energy                | death or recurrent       |
| appetite                | 7. feelings of        | suicidal ideation        |
| 4. Insomnia or          | worthlessness or      |                          |
| hypersomnia             | inappropriate guilt   | (Fried & Nesse, 2015).   |

Due to the heterogeneity of the disease, there are several subtypes of depression with different pathogenesis and treatment options, though their biological basis has yet to be fully

explained (Drysdale, 2017). Major Depressive Disorder (MDD) is one of the most prevalent risks to human life and productivity worldwide. Life expectancy for afflicted populations is estimated to be 7-10 years less than the general population, with a 1.8 fold mortality rate, partially due to increased risk of suicide (Otte, Gold, & Pennix, 2016). Clinical and public efforts to decrease suicide rates, though widespread, have been notoriously inconsistent, with less than a third of patients seeing clinical improvement (Thase & Rush, 1997).

The mechanism for human stress response is the hypothalamic-pituitary-adrenal (axis) (Figure 1). Upon encountering a stressor, (defined as any threat- physical or perceived, acute



**Figure 1.** Diagram of the major components of the Hypothalamic Pituitary Adrenal (HPA) Axis when facilitating a stress response in the human body. (Justin Salm '16)

or prolonged- to the well-being of an organism (Jacobson, 2014)) peptides corticotropin-releasing-hormone (CRH) and arginine vasopressin (AVP) (which serves to potentiate CRH function) are secreted by cells within the paraventricular nucleus of the hypothalamus, which in turn signal the release of adrenocorticotropic hormone (ACTH) from the pituitary

glands. This triggers the release of glucocorticoids, the workhorses of the stress response, from the adrenal glands. Glucocorticoids, namely corticosterone and cortisol in human endocrine systems, serve a variety of purposes, including mobilization of glucose, gene expression alterations, and negative inhibition of HPA-axis functionality (Mackin & Young, 2004). They primarily bind to two intracellular corticosteroid receptors within the cytosol,

the type I mineralocorticoid receptor (MR) and the type II glucocorticoid receptor (GR) (Klok, et al., 2011).

Mineralocorticoid receptors help to regulate electrolyte balance in the kidneys, where glucocorticoids are inactivated by 11-betahydroxysteroid dehydrogenase type 2 (11 $\beta$ HSD-2) (DeRijk & de Kloet, 2005). However, MR are primarily located in limbic areas of the brain such as the hippocampus, amygdala and hypothalamus and have a high affinity for cortisol binding (Herbert, 2013). Because of this, they are constantly saturated with cortisol, even during basal (non-stress) conditions, and function to regulate the amount of free plasma cortisol. A low affinity MR has recently been discovered, and though the role of this receptor has yet to be fully determined, this finding suggests that MR may play a larger role in stress response regulation than previously thought (Klok, et al., 2011). The low affinity GR (at a tenth of the affinity of high affinity MR) is present more ubiquitously than MRs throughout the brain and body and is highly concentrated in the hippocampus and prefrontal cortex (PFC), and becomes activated only when cortisol levels are particularly high due to stress response. GR can also bind at the peaks of the circadian cycle, and terminates extended HPA axis activity at several points through feedback inhibition of CRH, vasopressin and ACTH production (Velders, et al., 2011).

The HPA axis under basal conditions fluctuates in diurnal rhythms, governed by the hypothalamic suprachiasmatic nucleus, that vary in frequency and amplitude, with the highest peaks generally occurring in the morning approximately 45 minutes after waking (Sharpley, et al., 2016) (Jacobson, 2014). Alterations in these rhythms, particularly hyperactivity of the HPA axis and the resulting hypercortisolemia have been strongly linked to MDD as well as other disorders such as PTSD (Savic, Knezevic, Damjanovic, Antic, &

Matic, 2014), with hypercortisolemia present in about 50% of the depressed patient population, with even higher percentages for the melancholic depression subtype (Dougherty, Klein, Olino, Dyson, & Rose, 2009). Additionally, normalization of HPA activity has been found to precede clinical recovery (Holsboer, 2000) (Sarubin, et al., 2016).

This research suggests that elevated cortisol levels (in amplitude of cortisol pulses and not in frequency), particularly in the morning, can serve as a predictor of susceptibility to MDD and a marker for depression. (Herbert, 2013) (Jacobson, 2014). Hypocortisolemia, characterized by prolonged low concentrations of cortisol occurring primarily after periods of extreme elevation, has also been found to present in approximately 25% of patients with stress-related disorders (Sharpley, et al., 2016), and threatens lowered immune function. Both hypo- and hyper-cortisolemia have also been associated with metabolic syndrome (which presents with insulin resistance, abdominal obesity and high blood pressure among other symptoms) and prolonged high cortisol levels can cause Cushing's Syndrome, and patients with Cushing's Syndrome have increased incidence of depression (Vammen, et al., 2014), though that data may be correlational or be related to the impacts on quality of life by Cushing's itself. Research has linked metabolic syndrome and depression, with cortisol as the common factor, though results have been inconsistent as to the exact association. One study found that hypercortisolic, depressed patients are at increased risk for metabolic syndrome, suggesting that HPA axis hyperactivity and/or GR or MR abnormalities may be the link. However, this research has not yet been widely replicated (Vogelzangs, et al., 2009).

*HPA-axis modulation and neuroendocrine fluctuations in Depression*

Hyperactivity along the HPA-axis has been measured by observations of all locations of neuroendocrine activity along the axis. Depressive patients have been found to have several HPA changes including increase in CRH-secreting neurons in the limbic brain, increase in frequency and amplitude of ACTH secretory pulses and HPA dysregulation measurement via the CRH stimulation test and dexamethasone suppression test (DST) (Sher, 2003).

However, the clinical ability of the DST alone in depression diagnosis has been insufficient and is primarily used in diagnosis of Cushing's syndrome. Cortisol also interacts with brain functions that control mood, behavior, (PFC) as well as memory and recall (hippocampus), where it affects neurotransmitters and neuropeptides in brain circuits.

Because modulation of HPA axis activity is primarily controlled by corticosteroid receptors binding and negative feedback, they are essential targets of research for understanding HPA-axis dysregulation and prolonged glucocorticoid elevation. Other potential factors are p-glycoprotein membrane transporters that exclude glucocorticoids from cells, and type 1 and 2 isoforms of 11 $\beta$ -hydroxysteroid dehydrogenase (11  $\beta$  HSD) which modulate glucocorticoid levels via conversion of cortisol between its active state (cortisol) and inactive state (cortisone) 11  $\beta$  HSD inhibition results in increased ACTH, implying that activation of cortisone allows feedback inhibition of ACTH and HPA axis regulation (Jacobson, 2014). Twin research has found some evidence that elevated morning cortisol is moderately heritable, which suggests a genetic cause for HPA axis abnormalities.

#### *Single-Nucleotide-Polymorphisms in HPA-Axis Modulators*

Polymorphisms are common mutations throughout DNA, and consist of misplacement of nucleotides. Single Nucleotide Polymorphisms (SNPs), true to the name, are instances of

substitution of one nucleotide for another, and can have a multitude of effects depending on the nucleotides substituted, location on the gene, and what the gene is coding for. SNPs in genes that code for HPA axis and glucocorticoid regulation mechanisms can have varying degrees of alteration of hormone sensitivity and activity levels. Several SNPs have been identified that impact GR, MR and 11  $\beta$  HSD function.

### *$\beta$ HSD type 1 and 2*

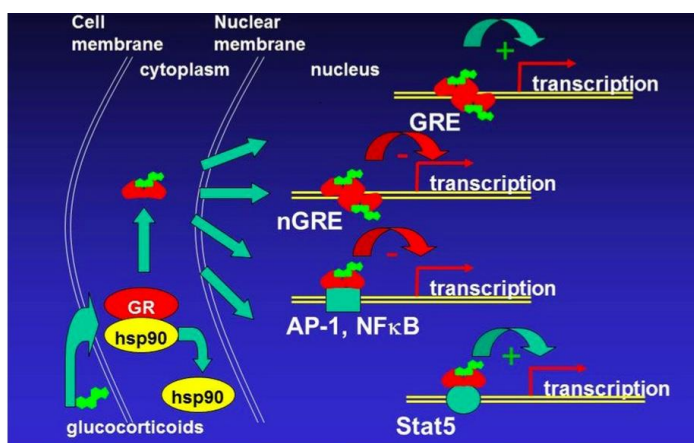
Ninety-five percent of cortisol is bound in the blood by corticosteroid-binding-globulin (CBG) as protection from degradation by liver P450 enzymes, but free cortisol passively diffuses across the membrane where 11  $\beta$  HSD type 1 (activation from cortisone to cortisol) and 2 (conversion from cortisol to the inactive cortisone) regulate cytosolic concentrations. Changes in function of these enzymes can significantly affect cortisol levels. 11  $\beta$  HSD type 1 is coded for by HSD11B1, and common SNPs have been associated in some studies with certain symptoms of metabolic syndrome, including hypertension, insulin resistance and type 2 diabetes (Fichna, et al., 2016).

### *Glucocorticoid Receptor Genotypes*

The GR is a ligand dependent transcription factor protein known as a nuclear receptor. The GR coding gene (NR3C1) is located on chromosome 5q21, and is approximately 150 kB long (Koper, van Rossum, & van den Akker, 2014) The GR protein itself is composed of three domains, the N-terminal transactivation domain (NTD), which recruits and binds transcription factors and co-regulators, a domain to which DNA binds (DBD) and a C-terminal domain for ligand-binding (LBD) (Kadmiel & Cidlowski, 2013). Without the presence of glucocorticoids the GR remains bound in the cytoplasm of the cell by chaperone



proteins and others that maintain the inactive state as well as accessibility for glucocorticoid attraction and binding. Upon ligand binding to the GR, it undergoes a transformational change and is translocated to the nucleus, where it induces or represses target gene expression through interaction with Glucocorticoid Response Elements (GREs) (Figure 2).



**Figure 2.** GR signaling pathway (Koper, van Rossum, & van den Akker, 2014, p. 64)

Glucocorticoid receptors are present in nearly every cell of the body, and have an incredibly diverse range of functions, as well as vast genetic variation due to an extensive list of identified SNPs. As of 2014 there were over 3000, and many more have been identified.

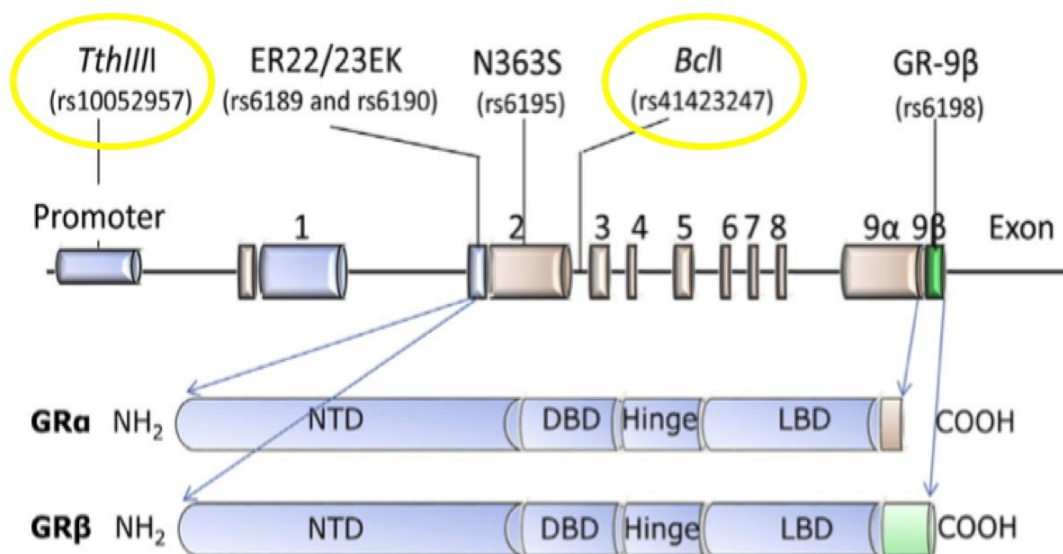
However, most of these have a minor allele frequency of less than 1% and therefore are statistically irrelevant.

A small number with higher minor allele frequency have also been found to alter glucocorticoid sensitivity (Koper, van Rossum, & van den Akker, 2014). Four of the most significant are Tth111I, ER22/23EK, N363S and BclI. TthIII1 (*rs10052957*) is a C → T substitution, and has been associated with elevated basal cortisol levels, but some research has suggested this effect is due to haplotype association with other GR SNPs, including ER22/23K. ER22/23EK (*rs6189* + *rs6190*) results from two single point substitutions on codons 22 and 23 and affects the secondary structure of the GR mRNA, favoring a less active start codon, which has been clinically associated with reduced GR sensitivity. N363S (*rs56149945*) is a A → G substitution, and has been associated with GR hypersensitivity and

enhanced cortisol suppression (Wust, et al., 2004). Lastly, BclII is characterized by a C → G substitution that has been associated with GR hypersensitivity (Koper, van Rossum, & van den Akker, 2014).

### *Mineralocorticoid Receptor Genotypes*

The MR is anatomically similar to the GR, but differs in its high affinity for binding glucocorticoids throughout the diurnal cortisol cycle, and is not as widely spread throughout the body as GR, being found primarily in the limbic brain and highly concentrated in the hippocampus. Because of its high affinity, the GR controls the onset and threshold of HPA axis stress response. There are two known significant MR SNPs, rs207 (*rs2070951*) and rs55 (*rs5522*) (Leeuwin, et al., 2011). Rs207 is a C → G substitution associated with higher morning cortisol levels (Mutz, Zyriax, Bondy, Windler, & Otte, 2011) while rs55 is an A → G substitution associated with MR reduced glucocorticoid sensitivity.



**Figure 3.** Glucocorticoid Receptor coding gene (NR3C1). Circled are the locations of the BclI and TthIII1 SNPs. Image obtained from [https://www.researchgate.net/figure/293801264\\_fig2\\_Fig-2-Glucocorticoid-receptor-gene-NR3C1-structure-and-functional-polymorphisms](https://www.researchgate.net/figure/293801264_fig2_Fig-2-Glucocorticoid-receptor-gene-NR3C1-structure-and-functional-polymorphisms)

**Table 1** GR, MR and 11BHS D glucocorticoid hypersensitivity or resistance SNP Oligonucleotides

Official name	Colloquial name	Phenotype	Gene Name	WT/MUT	Population Freq (minor allele, ie mutant)
rs41423247	Bcl I	Hypersensitivity	NR3C1	G/C	C=0.2546
rs56149945	N363S	Hypersensitivity	NR3C1	A/G	C=0.0208/2520
rs10052957	TthIII1	Resistance	NR3C1	A/G	A=0.2212/1108
RS12086634	RS120	Hypersensitivity	HSD11-B1	T/G	G=0.2066 (ExAC)
RS846910	RS84	Resistance	HSD11-B2	G/A	A=0.1010/506
RS2070951	RS207s	Hypersensitivity	NR3C2	C/G	G=0.4490/48980 (ExAC)
RS5522	Rs55s	Resistance	NR3C3	T/C	C=0.1188/14428 (ExAC)

## **METHODS**

### *Participants*

Buccal cheek cells were collected at Albany Medical College from 30 psychiatric patients, along with their scores for Beck Depression Inventory (BDI) as well as State and Trait Anxiety Inventories (STAI-S, STAI-T) and salival samples. Approval for this patient study was obtained from Albany Medical College's Institutional Review Board for patient samples. The purpose of the study and the rights of the patient were explained, and informed consent was obtained prior to sample collection. The patient was given a buccal swab with which they were instructed to rub their cheeks and gums for 15 seconds, then place swab-side first into a 15 mL centrifuge tube. Then they were instructed to deposit as much saliva as they could produce into a second 15 mL centrifuge tube. The tubes were then labeled with the patients' Top ID number and stored at 4 ° C until DNA extraction.

### *DNA extraction*

DNA was extracted from the buccal swabs as per the Environmental Health Perfect Protocol (107:517-520) (1999.) The swab handle was cut off and placed inside a 1.5 mL microcentrifuge tube, to which 600  $\mu$ L of 50 Mm NaOH was added. The tube was then closed and vortexed for 10 minutes, then heated at 95 ° C for 10 minutes in a heating block. 120  $\mu$ L 1M Tris (pH 8.0) was then added to the tube, and the brush was removed and discarded. The sample was then split into two tubes for storage, 100  $\mu$ L at 4 ° C and the remainder in the second tube at – 20 ° C.

### *Primer optimization*

Before genotypic analysis, SNP primers had to be optimized for allele-specific Polymerase Chain Reaction (PCR). Primers were first determined by identification of the SNP within the gene and production of wild-type specific, mutant specific and common primer sequences by a Web-Based Allele Specific Primer (WASP) Program. They were then ordered from Integrated DNA Technologies, Inc. (Skokie, IL) based on this WASP generation.

Primer function is dependent on several factors including temperature, cycle, Mg<sup>+</sup> presence and concentration, and the brands of the other PCR components, and required many trials manipulating these variables for each SNP in this study. Primer sets for two MR polymorphisms were optimized effectively- Bcl1 (*rs41423247*), a hypersensitivity SNP and TthIII (*rs10052957*) a resistance SNP.

#### **Bcl1 - hypersensitivity of the GR**

WT up 5' : AGTAGACAAGTTATGTCTGCTGACC  
 MT up 5' : AGTAGACAAGTTATGTCTGCTGACG  
 Com down 5' : AAATCAAACGAAAGCTGAAA

#### **TthIII1 - resistance of the GR**

WT r Primer 5' : AGATCAGGTCCTTGCCCTTGT  
 MT r Primer 5' : AGATCAGGTCCTTGCCCTTGT  
 Com Forward 5' : GAACTCCAGTGTGCCAGAAA

#### **Rs12086634 - hyperactivity of 11 $\beta$ -HSD 1**

Rs12086634.wt.r: GATGGCTATATTAAGAAATTC  
 Rs12086634.mut.r: GATGGCTATATTAAGAAATTA  
 Rs12086634.com.f: CTGTATCACTGCAGGT

#### **rs207 - hypersensitivity of the MR**

WT up Primer 5' : GTAGCCTTTGGTCTCCATTG  
 MT up Primer 5' : GTAGCCTTTGGTCTCCATT  
 Com down 5' : CAGATCTAAAGATATTATAG

**Figure 4** Output of WASP Generation. SNPs in bold were successfully optimized for the PCR protocol.

#### *PCR using allele-specific primers*

PCR occurs within a thermal cycler, utilizing multiple cycles of varying temperatures to denature DNA template and then replicate specific gene sequences. In allele-specific PCR,

primers included will only bind to the template if a certain allele is present, and therefore can be used to determine the presence of a certain SNP or wild-type allele. Three WASP generated allele-specific primers are included, including two forward primers with varying tails (WT and MT) and a common reverse primer. Each PCR reaction tube contained a total of 15 uL (table 2).

**Table 2.** *Reagent Quantity in PCR Reactions*

Reagent	Quantity (uL)
1) Deionized Water	5.125 ul
2) Buffer	1.5 ul
3) dNTPs	0.3 ul
4) Common Primer	1.5 ul
5) DNA taq polymerase	0.075 ul
6) WT or MUT Primer	1.5 ul
7) Q Solution	3 ul
7) Patient Template	2 ul

The PCR reactions were run in a Bio Rad C1000 Thermal Cycler. The conditions are shown in Table 3.

**Table 3.** *Thermal Cycler PCR Conditions for BclI and TthIII1 SNPs.*

	BclI	TthIII1
Denaturation Temperature	95°C	95 °C
Primer Annealing Temperature	56°C	61 °C
Primer Extension Temperature	72°C	72°C

### *Gel Electrophoresis*

To visualize PCR reaction products, 3 uL EZ vision dye was added to each of the PCR reactions, and they were run through a 2% agarose gel at 200 V for 40 minutes. The gel was then visualized under UV light to check for the presence of bands. The band size was compared against a 100 bp ladder. Example images of visualized successful and failed trials can be seen in Appendix A.

#### *Data Analysis & Statistical Testing*

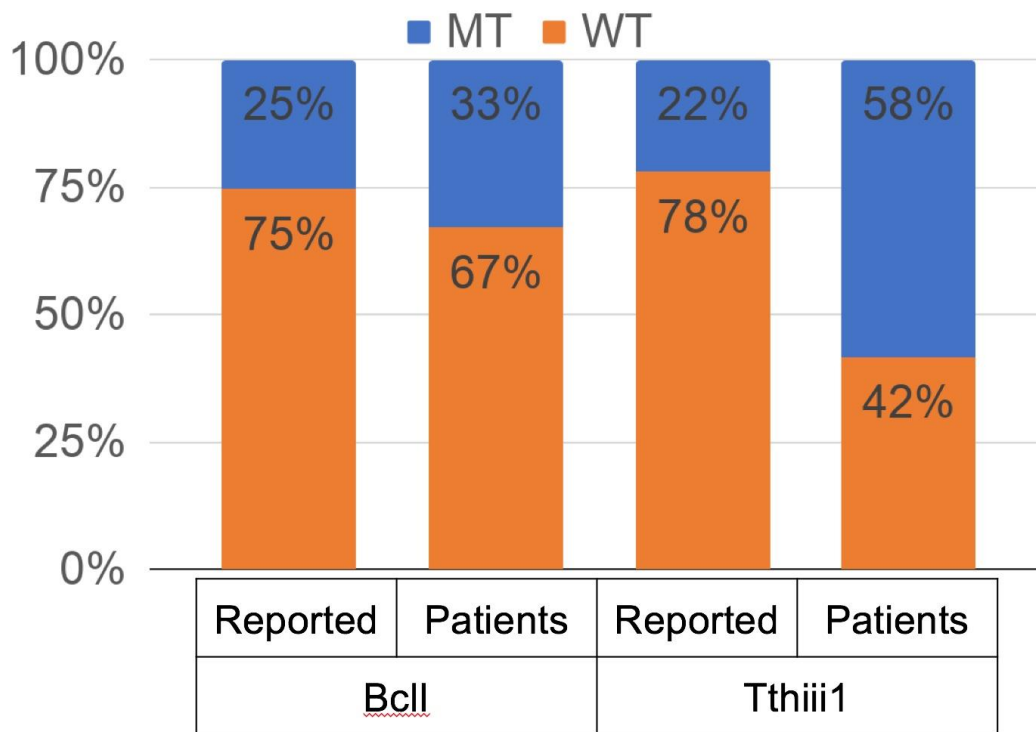
ANOVA analysis of polymorphisms and psychological testing information was performed using JMP v13 (SAS, Cary, NC).

Phone: 919.677.8000.

## **RESULTS**

#### *Prevalence of the SNPs*

Genotyping was determined for each of the patients for BCII and TthIII1 based on the protocols described above, and genotypic frequency of the alleles was compared to the reported population (figure 3). For the TthIII1 SNP, the psychiatric population had a higher MT allele frequency than the reported population, while BCII did not vary much between the populations, with a slightly higher prevalence of the MT allele.



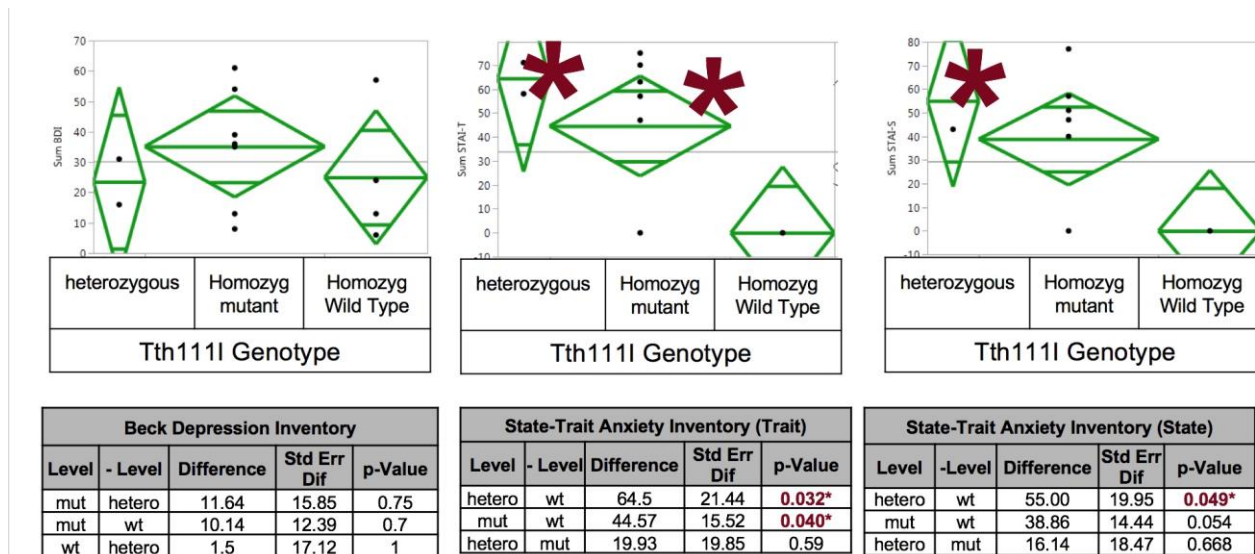
**Figure 5.** Genotypic frequency of psychiatric population compared to reported population for the Bcl1 and Tthiii1 SNPs.

*Associations between allelic frequencies and psychiatric parameters*

ANOVAs were performed using JMP with a  $p < 0.05$  significance criterion to compare genotypic frequencies for TthIII1 with psychiatric inventory scores for BDI, STAI-S and STAI-T. For the Trait Anxiety Inventory (STAI-T) significant difference was observed for patients carrying the MT allele (heterozygous or homozygous mutant genotype) compared to the homozygous WT sample, with scores higher with presence MT allele. For the State Anxiety Inventory, a significant difference was found between the heterozygous and



homozygous wild-type groups. There was no significant difference found for the Beck Depression Inventory between any of the groups ( $p > 0.05$ ) (figure 4).



**Figure 6.** Depression inventory scores were compared across genotypic groups. A significant difference was found between groups for STAI-T for patients that carry the mutant allele (heterozygous and homozygous mutant) and for STAI-S for the heterozygous group compared to the homozygous wild-type.

## DISCUSSION

The initial hypothesis was that the psychiatric population would have a lower allelic frequency of hyperactivity SNPs (BC11, N363S, RS207) and higher allelic frequency of resistance SNPs (TthIII1, RS84) compared to the population. As a result, it was hypothesized that for this sample, the resulting over-activity of the HPA axis will result in elevated basal cortisol levels in the psychiatric patient sample. These initial hypotheses failed to consider the fact that resistance and hypersensitivity SNPs have different effects on the HPA axis depending on the receptor that they code for, so while a higher allelic

frequency was found for the resistance SNP TthIII1, hyperactivity of GR SNPs may not result in higher basal cortisol levels. Additionally, tests were not performed for basal cortisol levels, so this hypothesis was unresolved. For TthIII1, however, these hypotheses are consistent with the literature and supported by these data.

#### *Genotypic frequencies*

The results suggest a difference in genotypic frequency of the TthIII1 MT allele between the psychiatric population and the normal population (figure 3), which suggests a possible role of the TthIII1 in modulating GR activity. These data are consistent with the implications of the literature associating TthIII1 with elevated basal cortisol levels (Wust, et al., 2004). As a GR SNP associated with resistance of the GR to binding cortisol, this mutation may result in less negative feedback inhibition of the GR on the HPA axis, resulting in greater overall cortisol production. The resulting hypercortisolemia has been strongly linked to psychiatric conditions such as MDD and PTSD (Sarubin, et al., 2016).

#### *Associations between SNP prevalence and psychiatric parameters*

Of note in the results was the finding of significantly higher differences in scores between genotypic groups for TthIII1 under State-Trait Anxiety Inventories, suggesting that the TthIII1 SNP may cause a difference in the presentation of anxiety within the disease, while there were no significant differences between any of the genotypic groups for BDI scores (figure 4). These results have concerning clinical implications, as cutoff values for depression diagnosis are based on sum-scores from BDI and other rating scales such as the Hamilton Rating Scale for Depression (HRSD), without consideration of the heterogeneity of the disease between patients.

Fried and Nesse (2015) argue that the pervasive use of sum-scores to estimate depression severity has stagnated progress in treatment of depression by obfuscating key insights into the presentation of the disease, such as biomarkers of depressive symptoms. Indeed, no biomarker has been found to be significant across the entire depressive population, though much research has been applied to identify this elusive depressive biomarker. A genome-wide association study with 34,549 subjects did not find a biomarker that was significant across the genome. As a result of this unsuccessful search, no biological tests have been added to depression criteria sets for the DSM-V. Fried and Nesse suggest that analysis of specific symptoms and more specialized scales for measurement of these symptoms can allow for personalization of care and therefore increased effectiveness of clinical treatment. The finding in the present study of significant differences between genotypic groups under a specific scale (STAI-T) with no significance under the sum-scale (BDI) supports this assertion (2015).

#### *Conclusions and Directions for Future Research*

Primer optimization trials were only consistently successful for the alleles of two SNPs analyzed above (Bcl1 and Tth111I), so genotypic analysis of other GR as well as MR and 11B-HSD SNPs was not complete for the patient population. The failure to optimize these primers is likely due in part to modifications of DNA isolation, PCR and gel electrophoresis protocol over trials to increase efficiency and clarity of products. Future research will use the improved protocol described above to optimize these alleles and generate a complete genotypic database for the psychiatric sample with which to conduct further statistical analysis.

Given the results of this study, a SNP of particular interest for further research is ER/EK. Though TthIII1 has been commonly associated with higher basal cortisol levels and altered promoter activity, some research suggests this effect may be due to haplotype associations with other GR SNPs such as Bcl1 or ER/EK (Koper, van Rossum, & van den Akker, 2014). Though no associations were found between genotypic frequencies of Bcl1 and TthIII1 for our population, suggesting an autonomous effect of TthIII1, the same cannot be determined for ER/EK given the current data set for our sample. Further, given the role of the GR in regulating HPA axis activity, basal cortisol levels may provide valuable insight on SNP function and differentiation. Basal cortisol levels for patient samples can be measured by cortisol assays, and quarterly follow up testing can track patient response to treatment, as normalization of HPA-axis activity has been found to precede clinical recovery (Holsboer, 2000) (Sarubin, et al., 2016). If, as the results suggest, polymorphisms such as TthIII1 affect inhibition of HPA-axis activity -- subsequently mediating glucocorticoid release-- differences in basal cortisol levels may be found between genotypic groups for these SNPs.

Lastly, these data emphasize the need for personalization of treatment based on disease heterogeneity. Future research should compare specific BDI Inventory scores between genotypic groups, to determine the extent to which depression sum scores conceal important symptomatic differences in depressive patients. Given clinical obfuscation of heterogeneity of the disease, the failure of biological research to identify significant biomarkers for the entirety of the depressive population is rendered unsurprising. Alternatively, research may be able to discover biomarkers for depressive groups separated by clinical symptomatic determination. These biomarkers can then serve as targets for drug development, increasing

effectiveness of pharmacological treatment and decreasing side effects that have been found to create severe and pandemic symptoms which further stagnate recovery of quality of life.

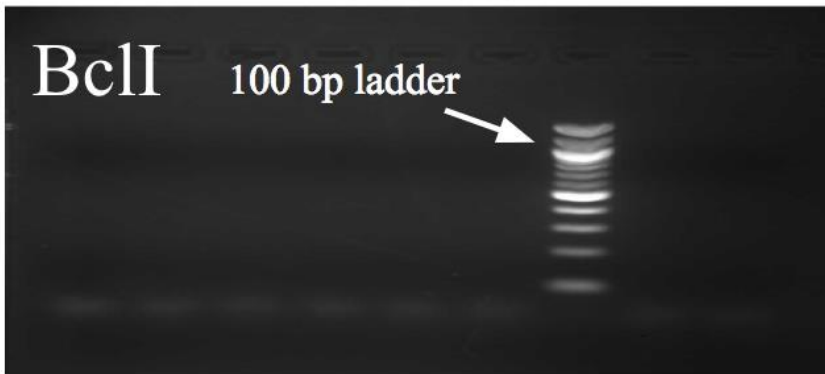
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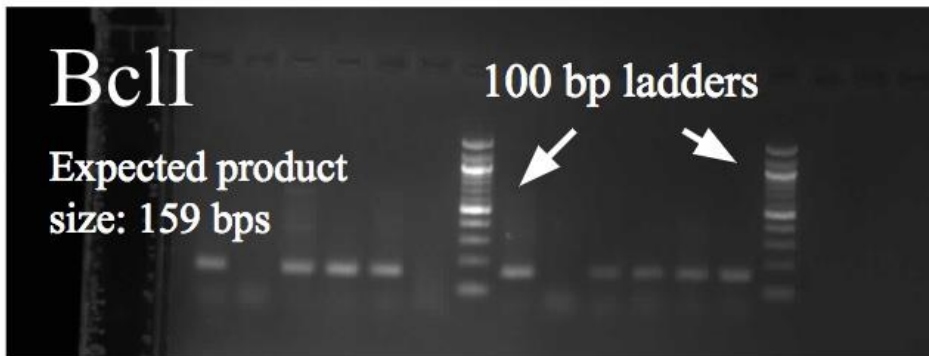
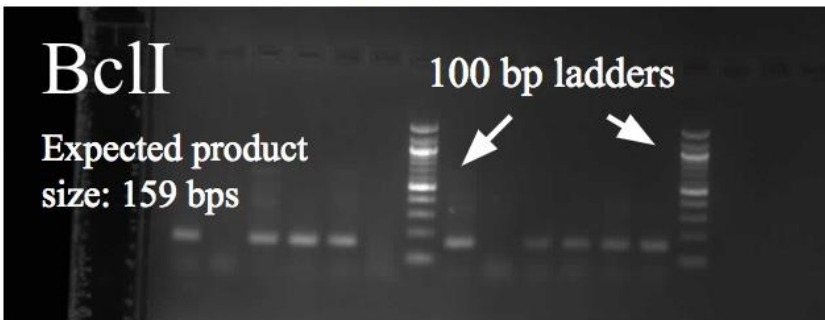
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**Appendix A: Gel Electrophoresis Trials and Genotyping**

Trial and Failure



Successful Trial



Subject	1		2		3		Lad	4		5		6		Lad
	WT	MT	WT	MT	WT	MT		WT	MT	WT	MT	WT	MT	
Band?	Y	N	Y	Y	Y	N		Y	N	Y	Y	Y	Y	
	↓		↓		↓			↓		↓		↓		
Determination	HOMOZ WT		HETERO		HOMOZ WT		Lad	HOMOZ WT		HETERO		HETERO		