6-2015

Lack of Glucocorticoid Receptor Hypersensitivity-Related Polymorphisms in an Undergraduate Population

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By

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Submitted in partial fulfillment

Of the requirements of

Honors in the Department of Biology

UNION COLLEGE

June, 2015

Introduction:

Obesity is a currently a major problem plaguing the world as a whole.[1] The International Obese Task Force (IOTF) estimates at least 1.1 billon adults are overweight with some 312 million of those obese, globally.[1] As of 2004, in less than twenty years, the prevalence of obesity had more than doubled in adults with childhood obesity prevalence rising even more rapidly.[1] Obesity, however, can be classified as a disease of its own or as a symptom of another disease. Two such diseases that have obesity as a symptom are Metabolic Syndrome and Cushing's Syndrome.[2,3,4,5]

Metabolic Syndrome is a condition that is represented by the presence of multiple risk factors. These factors, as indicated by The National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), include elevated waist circumference, elevated triglycerides, reduced high-density lipoprotein cholesterol (HDL-C), elevated blood pressure and elevated fasting glucose.[6] **Table 1** provides the modified categorical cut-off points for the five Metabolic Syndrome diagnostic criteria with the modifications including medical treatment received for diabetes, hypertension or elevated triglycerides.[2]

Table 1

Modified ATP III criteria for metabolic syndrome

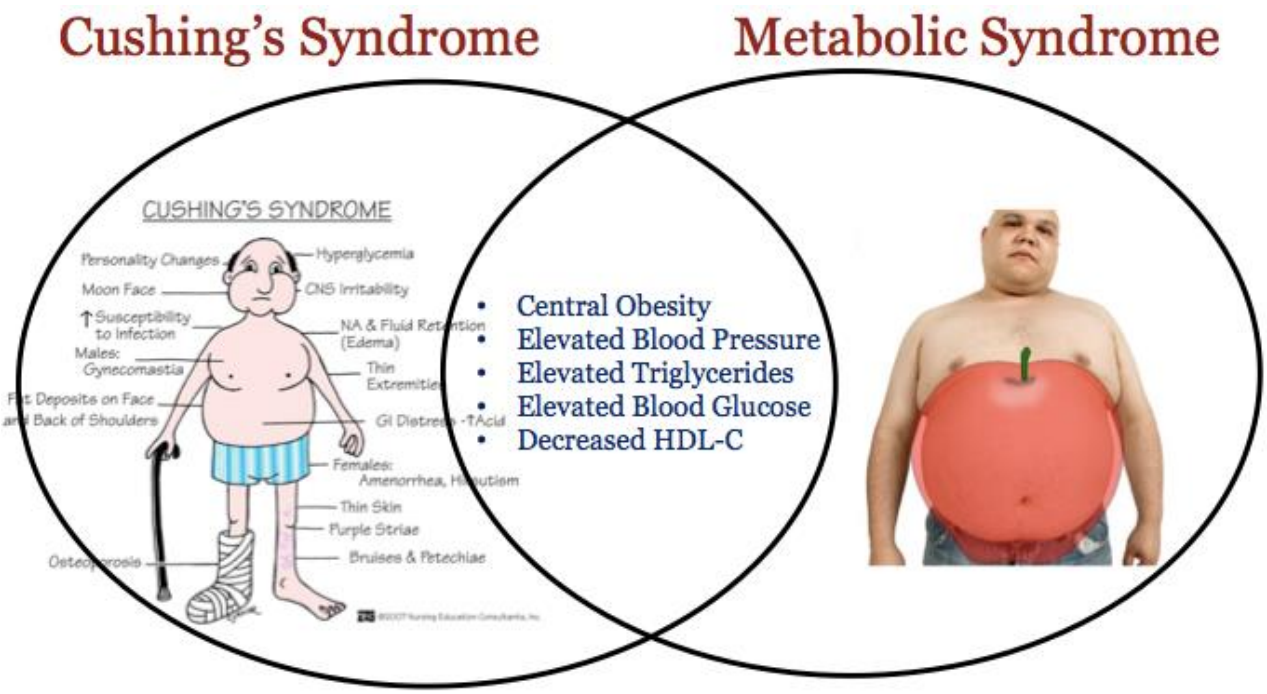
Risk Factor	Threshold criterion
Waist circumference, Women	> 35 inches (89 cm)
Waist circumference, Men	> 40 inches (102 cm)
Triglycerides	≥ 150 mg/dl (1.7 mmol/l) **OR** < 150 mg/dl + MED*
HDL cholesterol, Women	< 50 mg/dl (1.29 mmol/l)
HDL cholesterol, Men	< 40 mg/dl (1.03 mmol/l)
Systolic Blood Pressure	≥ 130 (mmHg) **OR** < 130 + MED*
Diastolic Blood Pressure	≥ 85 (mmHg) **OR** < 85 + MED*
Fasting Glucose	≥ 100 mg/dl (5.6 mmol/l) **OR** < 100 + MED*

*MED: triglyceride, blood pressure, or glucose lowering medications

Three of the five symptoms must pass the threshold criterion in order for a Metabolic Syndrome diagnosis to be established.[2] A diagnosis of Metabolic Syndrome comes with an increased risk for cardiovascular disease and type II diabetes mellitus.[7] While a diagnosis of Metabolic Syndrome increases risk of diabetes, once diabetes is present the subsequent risk for cardiovascular disease is increased even further.[7] Metabolic Syndrome with the presentation of insulin resistance is further associated with fatty liver, polycystic ovary syndrome, cholesterol gallstones, sleep apnea and lipodystrophies.[7] Metabolic Syndrome is becoming more common in society due to the increase in obesity rate; a Korean National Health and Nutrition Examination Survey found the prevalence of Metabolic Syndrome worldwide rose from 19.6% in 1998 to 32.4% in 2009.[4,7] In addition to the increased prevalence of cardiovascular disease, diabetes and the plethora of other health problems stemming from Metabolic Syndrome comes an influx in hospital visits and overall health care costs. Cardiovascular disease is the leading cause of death in the United States; in

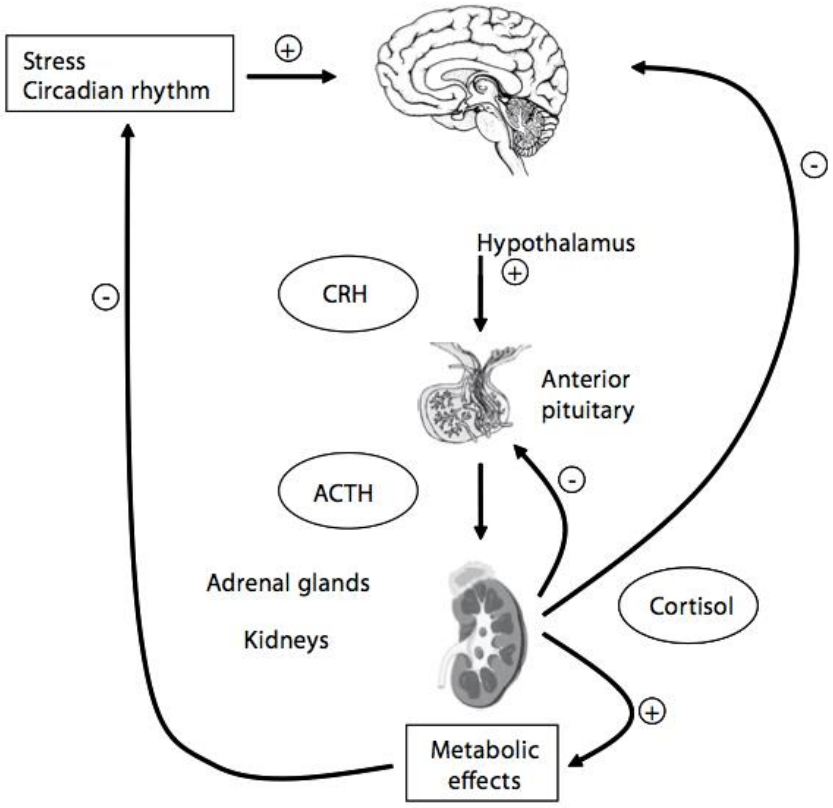
2011, 596,577 people died from cardiovascular diseases costing the US $273 billion dollars in 2010.[8,9] This accounts for some 17% of national health expenditures and these costs are expected to significantly increase to roughly $818 billion dollars in 2030.[8] While diabetes itself increases risk for health problems such as cardiovascular disease and stroke it is the leading cause of kidney failure, non-traumatic lower limb amputations and new cases of blindness in the US.[10] Diabetes was the seventh leading cause of death in the US in 2011; it directly killed 73,831 people and an additional 160,022 people had diabetes listed as a contributing factor to their deaths in 2007.[9,10] Diabetes cost the US $174 billion dollars in total in 2007.[10] These figures and statistics indicate the significance a diagnosis of Metabolic Syndrome has and the importance of continued Metabolic Syndrome research.

Cushing's Syndrome is a disorder with a broad clinical presentation that is characterized and diagnosed by hypercortisolism.[11] Cushing's can either be primary (Cushing's Syndrome) or secondary (Cushing's Disease). In the primary form the disorder is due to a defect at the site of cortisol production and is independent of the hormone stimulating cortisol's release.[3] The secondary form is dependent upon the hormone signaling cortisol's release and is a result of a defect not at the site of cortisol production.[3] As both result in hypercortisolemia, they will be referred to collectively as Cushing's Syndrome. Cushing's Syndrome's distinctive features include reddish or purple striae, facial plethora and rounding, dorsocervical fat pads, centripetal, or abdominal, obesity and proximal muscle weakness.[11,12] Many studies have acknowledged the phenotypic similarities between Cushing's Syndrome and Metabolic Syndrome.[2-4,12,13] Such similarities can be viewed in **Figure 1**.



Since the two syndromes have so many similarities, it has been hypothesized, by our lab and others, that in some cases Metabolic Syndrome represents a Cushing's Syndrome like state in the absence of elevated cortisol levels.[2] One potential mechanism by a Cushing's Syndrome like state could occur without elevated cortisol levels is if the glucocorticoid receptor has an elevated sensitivity to glucocorticoids as a result of one or more mutations to the glucocorticoid receptor.

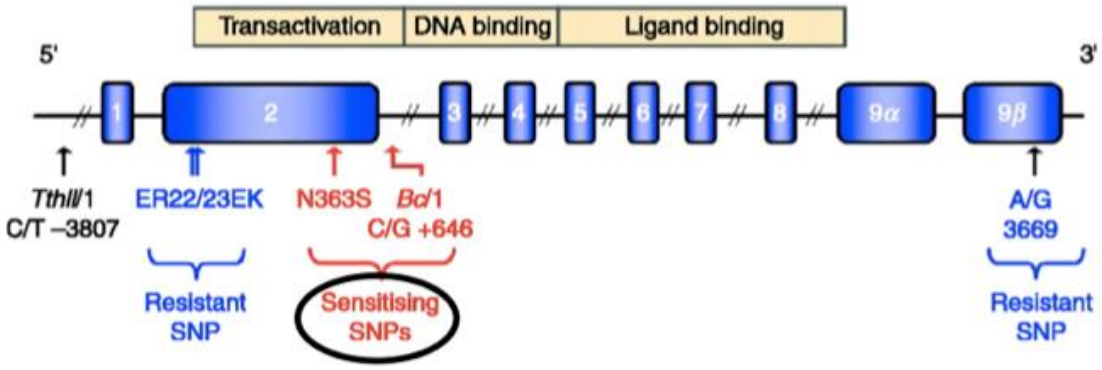
Cortisol is a steroid hormone, more specifically a glucocorticoid, which is produced in response to stress.[14] Its production is part of the hypothalamic-pituitary-adrenal (HPA) axis.[14] The HPA axis is seen in **Figure 2**.[15]



The hypothalamus in the brain responds to a stressor, either chronic or acute, by releasing corticotropin-releasing hormone (CRH).[16] CRH travels to the anterior pituitary gland where it stimulates the release of adrenocorticotropic hormone (ACTH), which subsequently travels to the adrenal cortex of the adrenal gland, specifically the middle layer of the cortex, the zona fasciculata, and promotes the release of cortisol.[16] Cortisol release is regulated by long-loop negative feedback whereby cortisol inhibits both the hypothalamus's release of CRH and the anterior pituitary's release of ACTH.[16] Cortisol's target is the glucocorticoid receptor and due to varying receptor sensitivities among individuals, the effects of glucocorticoids themselves vary greatly.[3] Cortisol's role, in addition to its immunosuppressant effects, is to increase blood glucose levels, central fat, bone resorption and arterial tone while decreasing muscle mass and bone formation.[3,4,14]

More broadly, it is responsible for insulin resistance, diabetes, dyslipidemia and cardiovascular disease, many of the diagnostic criteria for Metabolic Syndrome.[3] Evidence suggests a causal relationship between cortisol and obesity, specifically the abdominal obesity that is characteristic of both Metabolic Syndrome and Cushing's Syndrome.[2,3] Cortisol is the link that binds together Metabolic Syndrome and Cushing's Syndrome. In addition to abdominal obesity, chronic exposure to excess cortisol is responsible for the clinical phenotype of Cushing's Syndrome.[3]

The glucocorticoid receptor is an intracellular steroid hormone receptor coded for by the gene *NR3C1* located on chromosome five.[17] When not bound to cortisol, the glucocorticoid receptor is located in the cytoplasm of the target cell along with multiple heat shock proteins.[18] Once cortisol diffuses through the cell membrane and binds the receptor, the receptor homodimerizes and receptor and ligand translocate to the nucleus of the cell where it regulates gene transcription.[18] The bound receptor can either activate or repress gene expression, known as transactivation and transrepression, respectively, by interacting with glucocorticoid response elements within the promoter regions of the target genes.[18] The varying glucocorticoid receptor sensitivities among individuals are determined mostly empirically and several polymorphisms of the glucocorticoid receptor gene that alter glucocorticoid sensitivity have been identified as summarized in **Figure 3**.[3,14,19]



Two particular polymorphisms of the glucocorticoid receptor that positively correlate with hypersensitivity to cortisol, the asparagine to serine mutation at the 363rd codon of the receptor gene (N363S) as well as a mutation that abolishes the BclI site on the second intron of the receptor gene, positively correlate with characteristics of Metabolic Syndrome.[20]

The N363S mutation is a single nucleotide polymorphism (SNP) located at the 1220th nucleotide mutating an adenine to a guanine (AAT to AGT), thus making the 363rd codon serine as opposed to the wild type asparagine.[17,18] This mutation is associated with increased sensitivity to glucocorticoids *in vivo* as well as increased body mass index (BMI).[17,18,21,22] Further, the N363S mutation is associated with a tendency toward higher insulin resistance, increased blood pressure and decreased HDL-C levels.[17,18,22] One study reported the presence of the heterozygous N363S polymorphism in between 1.2 and 6.8% of a Caucasian population tested while another study found between 3.9 and 9.3% of a Caucasian population of European descent heterozygous for the mutation.[17,22] Australian individuals have among the highest prevalence of the polymorphism with studies reporting between 9.4 to 19.8% heterozygous for the mutation.[17,23] Allelic frequency of the N363S mutant allele has been reported to be around 6%.[15] It is clear that the prevalence of the polymorphism varies by ethnicity as two additional studies found the mutation was not

detected in a population of 192 Japanese individuals and an extremely low prevalence among a population of South Asian individuals. [17,23] All reported individuals with mutations were heterozygous; no homozygotes for the mutation were observed in any of three European populations.[23]

The mutation at the BclI site of the glucocorticoid receptor gene is also correlated with increased sensitivity to cortisol as well as abdominal obesity and insulin resistance. [18-20] The mutation is the loss of the BclI restriction site in the second intron of the glucocorticoid receptor gene 646 base pairs from the exon 2/intron 2 junction. [19] The mutation is a SNP transitioning a cytosine to a guanine. [18-20,23] One study has indicated a 27% allelic frequency of the G allele in their obese patients and another study found 9.7% of their population to be homozygous for the mutation with an additional 41.7% of their population heterozygous for the mutation. [20,23] One study noted that the BclI polymorphism occurs more frequently in a Caucasian population than in other ethnic groups. [20] Allelic frequency of the Bcl1 mutant allele has been reported to be around 35%. [15] Our lab hypothesizes that in a population of non-obese college students with normal range BMIs there will be a lower frequency of the mutant alleles for both the N363 as well as the BclI polymorphisms as compared to the general population.

Methods:

Subject Data

The study enrolled 8 undergraduate students, mostly white, with normal range BMIs. The mean BMI was 23.5 kg/m^2 with the standard deviation of the mean was calculated to be 4.7.

DNA Isolation

The experimental approach utilized in this investigation began with collecting cheek cells from the enrolled subjects. The cheek cells were obtained by a buccal wash; a saline solution was swished inside the subjects' mouths and returned to individual collection tubes. DNA extraction and isolation was then performed using a 10% CHELEX solution in 50mM Tris at 9.5pH. The cheek cells were pelleted in the collection tubes, the CHELEX solution was then added, the mixture vortexed, heated, cooled and again pelleted to retrieve the supernatant containing the subject's DNA.

Allele Specific Primers and DNA Amplification

Amplification of the DNA to identify the presence or absence of the N363S and BclI polymorphisms utilized an allele-specific polymerase chain reaction (PCR). That is, the artificial primers designed to anneal to the template strands and begin extension were designed to only anneal in the presence of either the wild type allele when reacting the template with the wild type allele-specific primer or the mutant allele when reacting the template with the mutant allele-specific primer. This specificity is seen in **Figure 4.**

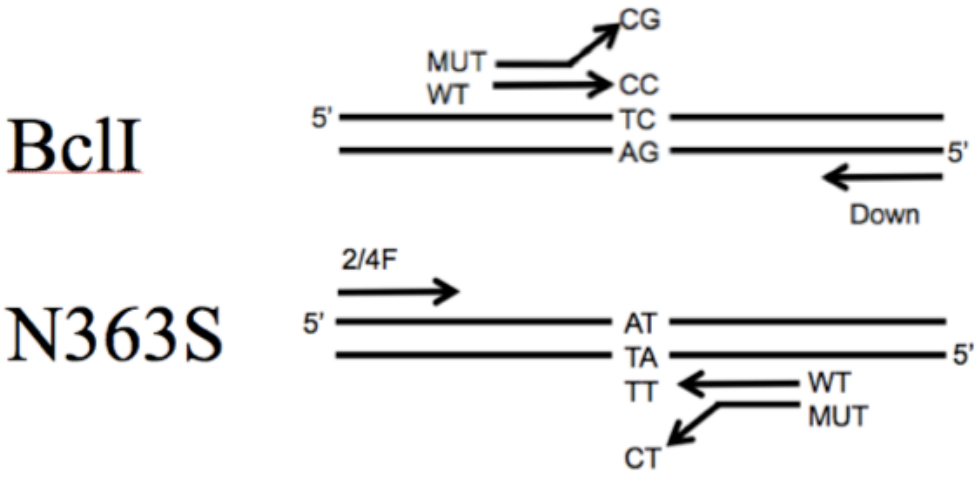


Figure 4. Allele-Specific Primer Polymerase Chain Reaction Diagram
Wild type, mutant and common primer location indicated using 5' → 3' arrows. Template displays wild type sequence. Primers include mismatch at penultimate nucleotide.

The Bcl1 and N363S sites in **Figure 3** include the penultimate and ultimate nucleotides present in the wild type sequence, therefore the indicated mutant primers will not anneal. To increase specificity of the reaction further, mismatched nucleotides at the penultimate positions of the allele-specific primers were introduced. The primer sequences are found in **Table 2**.

Table 2. Bcl1 and N363S PCR Primers

Name	Sequence (5' → 3')
N363S.2/4Forward	CCAGTAATGTAACACTGCCCC
N363S WT	AGATCCTTGGCACCTATTCCATT
N363S MUT	AGATCCTTGGCACCTATTCCATC
BclI WT	AGTAGACAAGTTATGTCTGCTGACC
BclI MUT	AGTAGACAAGTTATGTCTGCTGACG
BclI Downstream	AAATCAAACGAAAGCTGAAA

Using the specifically designed primers, PCR was used to amplify the target sequence. Each PCR reaction was 50µl total volume and contained water, 10x NovaTaq buffer with 15mM $MgCl^{2+}$, 10mM each dNTPs mix, *TAq* polymerase, common primer and one allele-specific primer. All PCR protocols include an initial 95°C denaturation step followed by 35 cycles of a 95°C denaturation, an annealing step, a 72°C extension. Both protocols included a final 72°C elongation step. The annealing temperature for the BclI reaction was optimized to 52°C while the annealing temperature for the N363S reaction was optimized to 56°C. PCR products were separated on a 2% agarose gel using electrophoresis and imaged using an ethidium bromide stain.

Results:

Through countless rounds of reaction optimization, the allele-specific primers properly annealed and showed specificity for the presence or absence of the BclI polymorphism for all 8 subjects' samples. The BclI results can be seen in **Figure 5.**

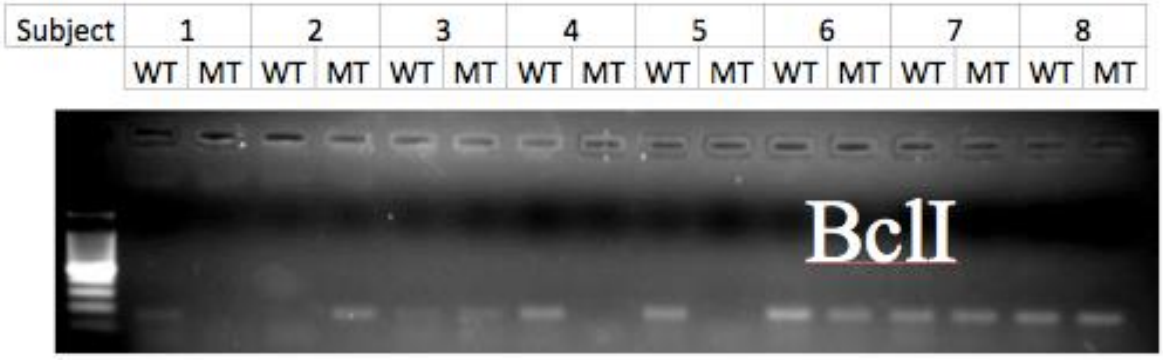


Figure 5. Detection of the BclI Polymorphism by Agarose Gel Electrophoresis
Genomic DNA was reacted with allele-specific primers (WT or MT) in a polymerase chain reaction for 35 cycles with the annealing temperature optimized to 52°C. Detection of genomic bands was conducted using 2% agarose gel electrophoresis, ethidium bromide staining and UV imaging.

Using the 100 base pair ladder in the leftmost lane as reference, the products can be seen to be 159 base pairs. The gel image indicates 3 homozygote wild type subjects, 1 homozygote mutant subject and 4 heterozygote subjects. This results in a 21% BclI mutant allele frequency within our sample.

For the detection of the N363S polymorphism, the reaction was only successful for 5 of the 8 subjects' samples. For the remaining 3 subjects' samples, no bands were detected using the standard imaging method utilized for the BclI reaction. The N363S results can be seen in **Figure 6.**

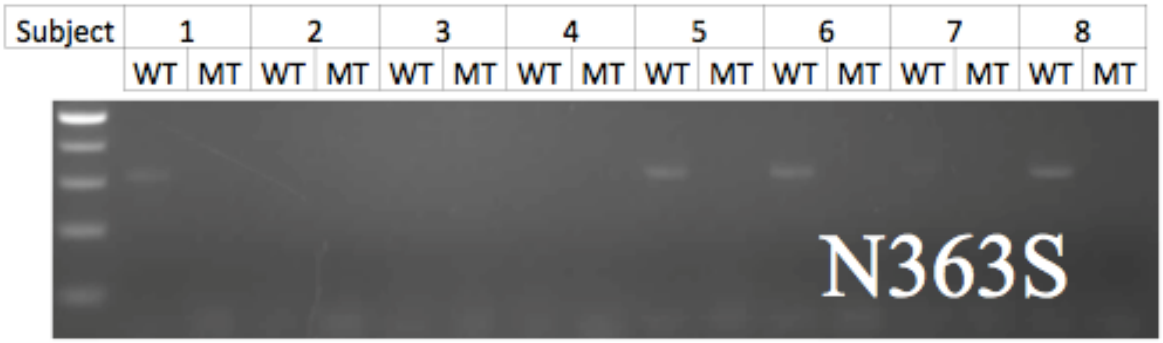


Figure 6. Detection of the N363S Polymorphism by Agarose Gel Electrophoresis
Genomic DNA was reacted with allele-specific primers (WT or MT) in a polymerase chain reaction for 35 cycles with the annealing temperature optimized to 56°C. Detection of genomic bands was conducted using 2% agarose gel electrophoresis, ethidium bromide staining and UV imaging.

Using the brightest band in the ladder as a 500 base pair reference, these bands corresponding to the amplified products can be seen to be 306 base pairs. The gel image indicates all 5 subjects' samples that successfully reacted to be homozygote wild type. The N363S mutant allele frequency is therefore 0% within our sample.

Discussion:

The results for the BclI mutation of 3 homozygous wild type subjects, 1 homozygous mutant subject and 4 heterozygous subjects as well as the results for the N363S mutation of 5 homozygous wild type subjects supports the lab's hypothesis that in a population of non-obese college students with normal range BMIs there will be a lower frequency of the mutant alleles for both the N363 as well as the BclI polymorphisms as compared to the general population. One study indicated that the mutant allele frequency for the N363S polymorphism is roughly 6% whereas the mutant allele frequency for the BclI polymorphism is roughly 35%. Our results of 0% and 21% mutant allele frequency for the N363S and BclI polymorphisms, respectively, therefore support the hypothesis.[15]

Throughout the optimization process, the N363S detection reaction presented as troublesome. This is further indicated by the dropout of 3 of the 8 samples reacted with N363S allele specific primers. These dropouts could be caused by any a number of reasons including, but not limited to, deterioration of the 3 genetic samples, an insufficient concentration of the genetic samples, an exceedingly high concentration of the genetic samples or simply an increased sensitivity of the template to the annealing temperature. The BclI reaction was successful in displaying specificity for all samples tested after the lengthy optimization process.

Both the BclI and N363S polymorphisms increase sensitivity to glucocorticoids, however the N363S polymorphism is located within an exon while the BclI polymorphism is found in an intron. As introns are noncoding sequences, it is not intuitive that a mutation here could effect subsequent transcription. The BclI polymorphism is only located 646 base pairs away from the intron/exon junction and therefore its likely disruptional effect is

on splicing. Another likely mechanism by which the BclI polymorphism could alter sensitivity is it being linked with other polymorphisms in more critical regions such as the promoter region or elsewhere. The polymorphism could introduce an insertion or a skipping effect to eliminate an adjacent exon from the RNA transcript. This could certainly affect responsiveness to glucocorticoids.

This study successfully validated that glucocorticoid receptor polymorphisms can be detected by allele specific PCR rapidly and inexpensively. This study was limited by its small sample size. Future research will focus on a much larger sample size and incorporate many other pieces of data to be analyzed along with the genetic screen. Already underway is collaboration with our lab at Union College and Ellis Hospital's Bariatric Surgery Group. Our lab hypothesizes that a population of patients presenting with Metabolic Syndrome will have an increased frequency of one or more of the hypersensitivity-causing polymorphisms than the patients without. Further, we intend to investigate the presence of a synergistic effect on the patients' phenotypes due to the presence of both N363S and Bcl1 polymorphisms. Further, our lab hypothesizes that the presence of glucocorticoid hypersensitivity will represent a predictive indicator for success after bariatric surgery as measured by rate of weight loss and ability of a patient to maintain weight loss. If glucocorticoid hypersensitivity correlates with failure to maintain weight loss, this could represent a novel avenue for understanding and ultimately treating these patients without performing revisional surgery. The implications of this investigation are great as genetic screening is becoming more popular as the technology to analyze Big Data improves. Such a detection method could lead to early detection of obesity risk factors and ultimately save lives.

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