Dopamine D2 Receptor Taq A1 allele predicts treatment compliance of LG839 in a subset analysis of pilot study in the Netherlands

Research Article

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Abbreviations: 2'-deoxynucleotide 5'-triposphates, (dNTPs); Allelic Discrimination, (AD); dopamine D2 receptor, (DRD2); dopamine, (DA); enkephalinase activity, (EKA); Feel Good Response, (FGR); polymerase chain reaction, (PCR); restriction fragment length polymorphism, (RFLP); Reward Deficiency Syndrome, (RDS); Society of Consumer Affairs Professionals, (SOCAP); Substance Use Disorder, (S.U.D.); ϒ-aminobutyric acid, (GABA)

Conflict of Interest: Kenneth Blum, B. William Downs, Lonna Williams and Roger Waite are all officers and stock holders of LifeGen, Inc., La Jolla, California, which has the worldwide exclusive license to manufacture and sell LG839. Brien Quirk is employed by Draco Natural Products Inc., San Jose California, distributors of Rhodiola rosea.

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Summary

Various types of individuals having “Reward Deficiency Syndrome (RDS)” related behaviors including sugar craving (e.g. obesity) have been described and heredity has been shown to be involved in some of these types. An important role of the mesolimbic dopamine system has been suggested in the reinforcing effects of a number of addictive substances (i.e. alcohol, nicotine, sugar etc) and recent molecular genetic studies are implicating the gene for the dopamine receptor (DRD2) as well as other genes in RDS and in particular obesity. We genotyped 1,058 Dutch subjects for polymorphisms of four candidate genes (PPAR gamma 2, MTHFR, 5-HT2a, and DRD2)
receiving the experimental DNA-customized nutraceutical LG839. In a subset of 27 subjects having a similar genotype pattern of the entire sample, and of all the outcomes and gene polymorphisms, only the DRD2 gene polymorphism (A1 allele vs. A2 allele) had a significant Pearson correlation with days on treatment ($r=0.42$, p=0.045). Compared to the DRD2 A1 carriers the number of days in treatment with LG839 was 51.9 ± 9.9 SE (95% CI, 30.8 to 73.0 ) and for the DRD2 A1 carriers the number of days on treatment with LG839 was 110.6 ± 31.1 (95% CI, 38.9 to 182.3 ). Thus the attrition was highest in the A1 genotype group. Thus, the genotype may be a predictor of treatment persistence and compliance. The feasibility of a pharmacogenetic approach in treating certain types of obesity related behaviors is cautiously suggested and warrants rigorous larger studies for confirmation.

I. Introduction

The obesity epidemic has extended the concern of obesity’s health consequences beyond cardiovascular disease and diabetes (Catalun et al. 2007). To evaluate the potential consequences of an experimental DNA-customized nutraceutical to reduce unwanted weight and outcome interactions with the dopamine D2 receptor gene (DRD2), we genotyped a self-identified obese population in the Netherlands. Current findings link obesity and reward sensations to the dopamine system and implicate dopamine genes in body weight, eating, and percentage of body fat (Chen et al., 2007a). Detailed consideration suggests that dopaminergic changes in the prefrontal cortex among individuals may increase their risk for obesity. Thus, individuals and populations with a high prevalence of the DRD2 A1 allele may experience higher rates of obesity in the presence of abundant food (Davis and Fox, 2008). From an evolutionary perspective, alterations in the dopamine system and/or brain reward cascade (Blum and Kozlowski 1990) appear to affect a wide range of behavioral phenotypes defined by Blum and colleagues in 1996 as “Reward Deficiency Syndrome” (RDS). Interestingly, it has been suggested that the dopaminergic D2 receptor may be a common link between obesity and drug addiction as evidenced via neuroimaging studies (Wang et al., 2004).

We suggest that recent evolutionary changes in the dopamine receptor genes may now be associated with increased food consumption in an obesigenic environment (Chen et al., 2007a) and polymorphisms of this gene may play a role in treatment outcomes involving neurochemical brain circuitry activation of dopaminergic pathways.

A. Experimental Rational

While there is a plethora of research on the utilization of nutritional approaches to obesity related health consequences there is a paucity of research involving neurotransmitter manipulation involving the brain reward system coupled with genetic polymorphic identification and obesity. In fact there over 26,083 studies on the relationship between obesity and nutrition; there are 4,258 studies on genes and obesity; there are 941 studies on brain neurotransmitters and obesity; there are 30 studies on the relationship of neurochemistry, obesity and genes; and there are no known studies concerning the relationship of neurochemistry, obesity genes and nutrition.

The common link between alcohol, heroin, nicotine and sugar craving provided the framework hypothesis for this study.

1. Mechanistic role of enkephalinase inhibition amino-acid precursor therapy in the brain reward cascade

Many years of research have established that pleasure-seeking has as its physiological basis the interaction of neurotransmitters in a cascade fashion centered in the mesolimbic structures of the brain (Blum and Kozlowski, 1990). The final pathway involves the activation of dopamine (DA) receptors in the N. Accumbens (NAC). It has been conjectured that at least four neurotransmitter systems are intimately involved in the so-called “Feel Good Response” (FGR). Therefore it is conjectured that serotonergic, opioidergic, GABAergic and catecholaminergic systems breakdown of synthesis, storage mechanisms, metabolic mechanisms, release at receptor(s) via genetic and/or environmental causes, could result in a lack of FGR. Certainly, animal research especially in the area of genetics reveal important biological substrates that seem to regulate intake of a variety of psychoactive substances through common biochemical pathways including dopaminergic activation of D2 receptors in the (NAC). In this regard a number of animal models including C57 vs. DBA mice; Sardian SP vs. SNP rats; P rats vs. NP rats, and AA vs. ANA rats all show reduced D2 densities in the mesolimbic areas in the preferring vs. the non-prefering animals (Liu et al., 2006).

While additional cases could be made for other neurotransmitter deficits, to date, the only molecular genetic defect, in terms of association with alcohol, drug , sugar seeking behavior (as well as other compulsive or neuropsychiatric disorders such as attention deficit hyperactivity disorder (ADHD)and Tourette Syndrome) which has been confirmed, are a number of variants of the DRD2 gene. A recent meta-analysis reveals an odds ratio for this association to be 2.18 with a p<10^-7. Supportive findings also include an effective sib-pair linkage study which also confirms this association in families with a p<0.002 for heavy drinking, and p<0.0002 for alcoholism (Noble, 2003).

Other work suggests that the variants of the DRD2 gene increase with severity of the disease (i.e., alcoholism and medical complications, cocaine addiction and family history of alcoholism, potency of the form of cocaine utilized and early deviant behavior) (Blum et al., 1990; Connor et al., 2002).
In support of this notion Comings and colleagues 1996 investigated the role of DRD2 gene polymorphisms and Substance Use Disorder (S.U.D.). To examine the possible role of genetic variants of the DRD2 gene in susceptibility to drug abuse they determined the prevalence of the TaqI A1 variant of the DRD2 gene in 200 white patients hospitalized in the Addiction Treatment Unit of a Veterans Administration Hospital. While the prevalence of the D2A1 allele was not significantly increased over controls, it did increase from 21% in subjects with alcohol abuse only to 32% in subjects with alcohol dependence only, consistent with other studies showing an association with the severity of alcoholism. By contrast, of 104 subjects with a discharge diagnosis of drug and alcohol abuse/dependence, 42.3% carried the D2A1 allele versus 29.0% of the 763 white controls (representing all white controls published to date) (p=0.006). Of those who spent more than $25 per week on two or more substances, 56.9% carried the D2A1 allele versus 28.2% of those abusing a single substance (p<0.0005). Multiple logistic regression analysis showed a highly significant association between multiple substance abuse based on money spent and the presence of the D2A1 allele (p=0.0003) and age of onset of abuse (p<0.0001). D2A1 carriers exceeded D2A2A2 subjects for a history of being expelled from school for fighting (p= 0.001), and of those ever jailed for violent crimes, 53.1% carried the D2A1 allele versus 28.8% of those jailed for non-violent crimes (p=0.011).

While DA is critical to maintain normalization of natural rewards the neuronal release of DA into NAc synaptic sites is somewhat complex. In 1989 our laboratory proposed an interactive cascade of events of mesolimbic function that lead to net DA release (Blum and Kozlowski, 1990). It was termed the “brain reward cascade” (Figure 1).

The interactions of activities in the separate subsystems above merge together into the much larger global system. These activities take place simultaneously and in a specific sequence, merging like a cascade. The end result is a sense of peace, pleasure, and well-being when these systems work normally. If there is a deficiency or imbalance, the system will work abnormally, causing the sense of well-being to be displaced by feelings of

![Figure 1. Brain Reward Cascade. Reproduced from Blum and Kozlowski, 1990](image-url)
anxiety, anger, low self-esteem, or other “bad feelings”. This can lead to the craving for a substance that masks or relieves those bad feelings such as carbohydrate binging, alcohol, or cocaine; or to other addictive behaviors such as compulsive gambling, compulsive sex, workaholism, or engaging in high risk activities.

In this cascade stimulation of the serotonergic system in the hypothalamus leads to the stimulation of delta /mu receptors by serotonin to cause a release of enkephalins. Activation of the enkephalinergic system induces an inhibition of γ-aminobutyric acid (GABA) transmission at the substantia nigra by enkephalin stimulation of μ receptors at GABA neurons. This inhibitory effect allows for the fine tuning of GABA activity. This provides the normal release of dopamine at the projected area of the n. accumbens (reward site of the brain). Both glutamate and cannibinoid receptors also play significant roles in mediating net DA release at the N. Accumbens.

A plethora of research has demonstrated that the reward sensation is related to complex cascade reactions involving several neurotransmitters and structures in the limbic system (Myers, 1989; Blum and Kozlowski; 1990). And that the ultimate result of the process is the activation of the mesolimbic dopamine pathway, which starts in the tegmental ventral area and ends in the dopamine D2 receptors on the cell membranes of neurons located in the NAc and the hippocampus.

The process, as described by Blum and Kozlowski in 1990 starts in the hypothalamus, with the excitatory activity of serotonin-releasing neurons. This causes the release of the opioid peptide met-enkephalin in the ventral tegmental area, which inhibits the activity of neurons that release the inhibitory neurotransmitter GABA. Both glutamergic and NMDA receptors are involved in this cascade.

The disinhibition of dopamine-containing neurons in the tegmental ventral area allows them to release dopamine in the nucleus accumbens and (via amygdala) in certain parts of the hippocampus, permitting the completion of the cascade and the development of the reward sensation. Usually, if the cascade is working properly, the reward, or the feeling of "well-being", or FGR is obtained, provided certain basic conditions are fulfilled.

Other work (Blum et al, 1983) from our laboratory is in agreement with the Maresh and colleagues in 1999, since we were the first to report on the alteration in alcohol intake in mice with a genetic predisposition to alcohol preference and known to have innate brain enkephalin deficiencies. We have been able to significantly attenuate both volitional and forced ethanol intake respectively by acute and chronic treatment with hydrocinnamic acid and D-phenylalanine, known carboxypeptidase (enkephalinase) inhibitors. The role of D-Phenylalanine as an enkephalinase inhibitor was studied by Marcello and colleagues who showed in 1996 that D-phenylalanine decreased "enkephalinase" activity (EKA) in plasma and CSF in humans. Since these agents, through their enkephalinase inhibitory activity, raise brain enkephalin levels, we proposed that excessive alcohol intake can be regulated by alteration of endogenous brain opioid peptides. In a series of experiments the role of amino precursor therapy and enkephalinase inhibition resulted in significant anti-craving benefits with regard to substance seeking behavior including sugar binging (Blum and Braverman, 2000).

Table 1 provides a list of proposed ingredients for the Synapatamine Complex (SG8839).

Based on the previous work involving the relationship between opioid peptides, narcotic antagonism and opiate and alcohol treatment, (Chen et al, 2004) our laboratory decided to test the hypothesis that possibly by combining a narcotic antagonist and amino-acid therapy consisting of an enkephalinase inhibitor (D-phenylalanine) and neurotransmitter precursors (L-amino-acids) to promote neuronal dopamine release might enhance compliance in methadone patients rapidly detoxified with the narcotic antagonist Trexan (Dupont, Delaware).

Thanos and colleagues found in 2001 increases in the DRD2 via adenosoral vector delivery of the DRD2 gene into the NAc, significantly reduced both ethanol preference (43%) and alcohol intake (64%) of ethanol preferring rats, which recovered as the DRD2, returned to baseline levels. This DRD2 overexpression similarly produced significant reductions in ethanol non-prefering rats, in both alcohol preference (16%) and alcohol intake (75%). This work further suggests that high levels of DRD2 may be protective against alcohol abuse. The DRD2 A1 allele has also been shown to associate with heroin addicts in a number of studies. This may have relevance to other addicting substances such as cocaine (Thanos et al, 2008) and possibly sugar.

In addition, other dopaminergic receptor gene polymorphisms have also associated with opioid dependence. For example, Kotler and colleagues showed in 1997 that the 7 repeat allele of the DRD4 receptor is significantly overpresented in the opioid-dependent cohort and confers a relative risk of 2.46. This has been confirmed by Li and colleagues in 1997 for both the 5 and 7 repeat alleles in Han Chinese case control sample of heroin addicts. Similarly Duaux and colleagues in 1998 in French Heroin addicts, found a significant association with homozygotes alleles of the DRD3-Bal 1. A study from NIAAA, provided evidence which strongly suggests that DRD2 gene is a susceptibility gene for substance abusers across multiple populations (Xu et al, 2004).

Moreover, there are a number of studies utilizing amino-acid and enkephalinase inhibition therapy showing reduction of alcohol, opiate, cocaine (also Methamphetamine) and sugar craving behavior in human trials (Blum et al, 2007a; Chen et al, 2007b). Over the last decade, a new rapid method to detoxify either methadone or heroin addicts utilizing Trexan sparked interest in many treatment centers throughout the United States, Canada, as well as many countries on a worldwide basis. In using the combination of Trexan and amino-acids, results were dramatic in terms of significantly enhancing compliance to continue taking Trexan (Chen et al, 2004). The average number of days of compliance calculated on 1000 patients, without amino-acid therapy, using this rapid detoxification method is only 37 days. In contrast, the 12 subjects tested, receiving both the Trexan and amino-acid therapy was
relapse-free or reported taking the combination for an average of 262 days (p<0.0001).

Thus, coupling amino-acid therapy and enkephalinase inhibition while blocking the delta-receptors with a pure narcotic antagonist, may be quite promising as a novel method to induce rapid detox in chronic methadone patients. This may also have important ramifications in the treatment of both opiate and alcohol-dependent individuals, especially as a relapse prevention tool. It may also be interesting to further test this hypothesis with the sublingual combination of the partial opiate mu receptor agonist buprenorphine or combining this regimen with CB1 receptor antagonist rimonabant only in the short term. The CB1 receptor blocker rimonabant has been shown to be effective in the short term therapy of obesity in 289 studies.

With this in mind substituting drugs with sugar, we evaluated the interaction of the experimental DNA-customized weight management agent LG839 with a number of reward and obesity candidate genes.

**II. Methods**

**A. Participants**

The first stage of the study involved a broad sample of 1,058 subjects who had self-identified themselves as obese or overweight. All subjects were recruited from customers who had purchased LG839 (Genotrim Variant) as part of a commercial pilot in the Netherlands from January 2006 to February 2007, and study participants were approached without a particular schema. The study subjects were primarily an ethnically homogenous group of Dutch decent. The second stage of the study involved a narrow sample of 27 subjects (there were nine males and eighteen females) who had self-identified themselves as obese or overweight. In this regard the beginning start weight for 26 of the participants was a mean of 105.93kg +/- 17.80.

Table 1. Amino acid nutrition therapy

<table>
<thead>
<tr>
<th>Supplemental Ingredient</th>
<th>Restored Brain Chemical</th>
<th>Addictive Substance Abuse</th>
<th>Amino Acid Deficiency Symptoms</th>
<th>Expected Change</th>
<th>Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Phenylalanine or DL-Phenylalanine</td>
<td>Enkephalins, Endorphins</td>
<td>Heroin, Alcohol, Marijuana, Sweets, Starches, Chocolate, Tobacco</td>
<td>Most Reward Deficiency Syndrome (RDS) conditions sensitive to physical or emotional pain. Crave comfort and pleasure. Desire certain food or drugs. D-phenylalanine is a known enkephalinase inhibitor.</td>
<td>Reward stimulation. Anti-craving. Mild anti-depression. Miled improved energy and focus. D-Phenylalanine promotes pain relief, increases pleasure.</td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine or L-Tyrosine</td>
<td>Norepinephrine, Dopamine</td>
<td>Caffeine, Speed, Cocaine, Marijuana, Aspartame, Chocolate, Alcohol, Tobacco, Sweets, Starches</td>
<td>Most Reward Deficiency Syndrome (RDS) conditions. Depression, low energy. Lack of focus and concentration. Attention-deficit disorder.</td>
<td>Reward stimulation. Anti-craving. Anti-depression. Increased level of energy. Improved mental focus.</td>
<td></td>
</tr>
</tbody>
</table>

Rhodiola rosea has been added to the formula and is a known Catechol-O-methyl transferase inhibitor (COMT) . This provides more synaptic dopamine in the VTA /NAc. (Mattioli L, Perfumi M Rhodiola rosea L. extract reduces stress- and CRF-induced anorexia in rats.J Psychopharmacol. 2007 21:742-50.)

Chromium salts – This has been added to the formula to enhance brain concentrations of serotonin. Note: To assist in amino-acid nutritional therapy, the use of a multi-vitamin/mineral formula is recommended. Many vitamins and minerals serve as co-factors in neurotransmitter synthesis. They also serve to restore general balance, vitality and well-being to the Reward Deficiency Syndrome (RDS) patient who typically is in a state of poor nutritional health. The utilization of GABA is limited due to its polar nature and ability to cross the blood brain barrier and Glutamate is used in a low level only to prevent over-inhibition of enkephalin breakdown and subsequent inhibition of GABAergic spiny neurons of the substantia nigra.
While this would suggest obesity in these subjects we did not have their BMI’s. These subjects participated in the LG839 pilot in the Netherlands, were willing to complete a retrospective survey about their results, and were willing to participate without compensation (Table 2).

Table 2. DNA-Customization example of the intervention (Reproduced from Chen et al, 2007b with kind permission from Springer).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ingredient</th>
<th>Serving</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine Receptor</td>
<td>Synaptamine Complex (DL-</td>
<td>1725 mg</td>
<td>The ingredients in Synaptamine include: (1) D-Phenylalanine – D-Phenylalanine inhibits enkephalinase, the enzyme that metabolizes or breakdown enkephalins, thereby increasing the availability of enkephalins and, presumably, making more dopamine available at the reward sites especially under stressful conditions. [Blieki-Gorzo et al. 2008] (2) L-Phenylalanine – L-Phenylalanine stimulates the production of dopamine, and/or increase norepinephrine levels in the reward area of the brain. The major problem with this amino acid is that it could compete with other amino-acids such as blood born L-tryptophan and L-tyrosine at the large neutral amino-acid brain carrier system. [2] However, other data demonstrate for the first time, that the synthesis and release responses to some dopaminergic agents may be elicited from synaptosomal dopamine, which is formed by the hydroxylation of phenylalanine. Amphetamine and Cogentin increased the release of dopamine formed from 14C-phenylalanine in rat caudate nucleus synaptosomal preparation and concomitantly stimulated the synthesis. Amfotelic acid also caused a net release of that dopamine. In conclusion, the results suggest that synaptosomal particles represent a unit capable of synthesizing dopamine from L-phenylalanine and that synthesis from this precursor may be under the regulatory control of the particles. However, Tyr is the preferred substrate; consequently, unless Tyr concentrations are abnormally low, variations in Phe concentration do not affect catecholamine synthesis. Unlike Tyr, Phe does not demonstrate substrate inhibition. Hence, high concentrations of Phe do not inhibit catecholamine synthesis and probably are not responsible for the low production of catecholamines in subjects with phenylketonuria. [Fernstrom &amp; Fernstrom, 20007] (3) L-glutamine – L-glutamine increases brain GABA levels at receptors associated with anxiety. Its major use is to maintain balance in case of over inhibition by D-phenylalanine. (4) L-Tyrosine – L-Tyrosine increases brain dopamine levels as a direct precursor amino-acid synthesis promoter. Its major use is for the repletion of utilized and thereby metabolized neurotransmitter (i.e. dopamine). (5) L-5-hydroxytryptophan - The effect of systemic administration of 5-hydroxy-L-tryptophan on the release of serotonin in the lateral hypothalamus of the rat in vivo was examined utilizing brain microdialysis. Administration of 5-HTP caused an immediate increase of the 5-HT in dialysates, which was long lasting and dose dependent. When calcium was omitted from the perfusion medium, thereby limiting exocytosis, levels of basal 5-HT were significantly decreased and the 5-HTP–induced response of 5-HT was markedly attenuated. (6) Pyridoxal-5-phosphate – Pyridoxal-5-phosphate is the active ingredient of vitamin B6 to serve as a co factor in the production of neurotransmitters and to enhance the gastrointestinal absorption of amino acids. (7) Chromium Salts (Nicotinate and Picolinate) - A novel oxygen-coordinated chromium nicotinate has been shown to promote brain serotonin production. In addition other chromium salts, such as the picolinate, has effects on serotonin production. A major effect of brain serotonin is that it is considered an appetite neurotransmitter. With the DRD2 mutation, a subject is prone to Reward Deficiency Syndrome and thus requires a greater amount of Synaptamine to overcome the predisposition to reduced DRD2 receptor sites.</td>
</tr>
<tr>
<td>A2/A2</td>
<td>Synaptamine Complex (DL-phenylalanine, Chromium, L-glutamine, L-tyrosine, L-5-HTP, Vitamin B6 [pyridoxal-5-phosphate])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>Synaptamine Complex (DL-phenylalanine, Chromium, L-glutamine, L-tyrosine, L-5-HTP, Vitamin B6 [pyridoxal-5-phosphate])</td>
<td>2,750 mg</td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>Synaptamine Complex (DL-phenylalanine, Chromium, L-glutamine, L-tyrosine, L-5-HTP, Vitamin B6 [pyridoxal-5-phosphate])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1/A2 or Homozygous A1/A1</td>
<td>Synaptamine Complex (DL-phenylalanine, Chromium, L-glutamine, L-tyrosine, L-5-HTP, Vitamin B6 [pyridoxal-5-phosphate])</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
B. Study design
This cross-sectional, observational study included a genetic analysis involving a panel of genes, along with one evaluation questionnaire occurring in January 2007 providing two key self-reported retrospective data sets on the subjects’ health before taking LG839 and then after taking LG839. Self-reported evaluations were collected through an online questionnaire disseminated over email to study participants. The primary objective was to evaluate the safety and efficacy of LG839 in obese and overweight subjects, and specifically to look at differences in weight, mood, sleep, digestion, and other health issues before and after taking LG839. The results of the weight loss effects of the Genotrim® variant [a former variant of LG839] are a subject reported elsewhere (Blum et al., 2006, 2007b). All participants provided written consent, and the study protocol was approved by the institutional review board of the non-profit research organization, PATH Medical Foundation, located in New York, NY, USA. Data was collected online independently by LooseFoot Computing Ltd. of Regina, Saskatchewan, Canada.

C. Laboratory Measurements
The laboratory testing was performed in a high-complexity, CLIA-certified laboratory known as PRIMEX Clinical Laboratories under contract with a nutritional company of San Diego, California, USA.

1. DRD2
All subjects were genotyped based on a neutral identification number and read without knowledge of the individual being typed. Total genomic DNA was extracted from each coded blood sample, and aliquots were used for polymerase chain reaction (PCR) analysis. The oligo-nucleotide primers 5’-CGGTCACCCCTTCAAGTGTATCTA-3’ and 5’CCGTCAGCGCTTGGCAGATGTCTA-3’ were used to amplify a 310-base pair (bp) fragment spanning the polymorphic Taq1A site of the DRD2 gene. The DA1 and DA2 genotyping were performed by a PCR technique (Blum et al., 1990; Comings et al., 1996, 2000). PCR was performed in 30-μL reaction mixtures containing 1.5 mM MgCl2, 20 mM 2’-deoxynucleotide 5’-triphosphates (dNTPs), 0.5 μM primers, 1 μg of template DNA 1, 5U of Taq polymerase (Boehringer Mannheim Corp., Indianapolis, IN), and PCR buffer (20 mM Tris-HCL [pH 8.4] and 50 mM KCl. After an initial denaturation at 94°C for 4 minutes, the DNA was amplified with 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C, followed by a final extension step of 5 minutes at 72°C. The PCR product was digested with 5 U of Taq I for 22 hours at 65°C for the Taq1A polymorphism. Digestion products were then resolved on a 3% agarose gel (5%cm) containing 0.65 μg/ml ethidium bromide. There were three DRD2 Taq1A genotypes: 1) the predominant homozygote A2/A2, which exhibits two restriction fragments of 180 and 130 bp; 2) the heterozygote A1/A2, which exhibits three restriction fragments of 310, 180, and 130bp; and 3) the rare homozygote A1/A1, which produces only the uncleaved 310-bp fragment (Comings et al., 1996).

2. MTHFR
Genotyping for the MTHFR C677T polymorphism was performed by the polymerase chain reaction (PCR) technique and restriction fragment length polymorphism (RFLP) analysis. We designed PCR primers 5’-CCACACCTATCTGTTTATATCC-3’ (sense) and 5’-TGGGAGAATCTACGGACT-3’ (antisense) with DNASIS Pro Ver 2.0 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Since the C to T transition at nucleotide 677 produces a Hinf I digestion site, the amplified 469-bp product derived from the mutant gene was cleaved into 393-bp and 76-bp fragments by Hinf I (TaKaRa Bio Inc., Shiga, Japan), which leaves the wildtype gene unaffected. After electrophoresis through 6% polyacrylamide gel, the digestion products were visualized by staining with ethidium bromide.

3. 5-HT2A
Genotyping of the -1438G/A polymorphism of the 5-HT2A gene was carried out by polymerase chain reaction (PCR) and restriction digestion as described previously. Genomic leukocyte DNA (100 ng in a final volume of 10 μL) was amplified by PCR using the following primers: 5’-AACAGCTGAGTGCAACAGC-3’ and 5’-AACAAACTTATATCTACCCAC-3’. The primers amplified a product of 468 bp. The PCR conditions were as follows: an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 30 seconds, with a final extension of 10 minutes at 72°C. The PCR reaction product was digested at 37°C overnight with 5 U of the restriction enzyme MspI (New England Biolabs, Beverly, MA). The -1438G allele was cut into 244-bp and 224-bp fragments, whereas the -1438A allele remained undigested. The fragments were separated on a 2% agarose gel.

4. PPAR-Gamma
Two hundred base pairs of sequence surrounding PPAR2 Pro12Ala was provided to Applied Biosystems (Foster City, CA) to develop Taqman Allelic Discrimination (AD) Assays using their assay by design platform. Genotyping of the Pro12Ala AD was performed using primers (0.9 mol/L each) Forward 5’-TTATGGTGAAACTCTGGGAGATT-3’ and reverse 5’-TGGACAGTGTATCACAGTTAGGG-3’ and the Taqman MGB probes Fam- TTCTGGTGCAATGAG Vic- CTCTGCGGCATAG (0.1 mol/L each; Applied Biosystems). Four microliters of a 10 ng/L stock of DNA was dispensed into 384-well PCR plates using a Biomek FX robot (Beckman Coulter), to which 6L of a mix containing primers, MGB probes and TaqMan Universal PCR Master Mix (Applied Biosystems) were added. These were sealed with optical seals (Applied Biosystems) and incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min before analysis on a 7900HT plate reader (Applied Biosystems). Individual genotypes were determined using SDSv2.1 software (Applied Biosystems).

C. Nutraceutical Customization
Based upon the genetic profiles derived, certain ingredients in LG839 were customized. In this study, we customized several ingredients based upon genotype. Table 2: outlines one example of the DNA-customization of an ingredient complex Synaptamine involved in LG839.

D. Measurements of Efficacy and Safety
Separate from the laboratory measurements, all measurements of efficacy and safety were self-reported by the study subjects using a one-time retrospective online questionnaire. To monitor persistency, we asked study subjects to self-report on their frequency of compliance to the product’s serving instructions. Inherent to the nature of self-reported data collection, there are strengths and weaknesses that cannot be quantified.

E. Statistics
The means and standard deviations were calculated before and while on LG839 for each item in the questionnaire. To test for a change, the paired t-test was used on the change score, with
change calculated as while response minus the before response for each item. Significance was denoted at each level of 0.05, 0.01 and 0.001 in the results. Due to the small number of subjects tested the possibility of spurious results must remain a question until a larger sample size could be obtained in future controlled studies. The Statistical Analysis System was the software used (SAS Institute, version 9.1 for the PC, Cary, North Carolina, 2006) for these calculations.

Two sample t-tests were used with Satterthwaite’s approximate t-test when the F-test for equal variances rejected for comparing changes in each item for those with each genetic polymorphism compared to those without the polymorphism.

Genotypic and allelic distributions were compared with the Pearson chi-square test for homogeneous variances. Three groups were compared, the historic controls, the sample of 1,058 and the sample for this analysis, n=27. The effect of each polymorphism on quantitative variables was tested using multiple linear regression. Pearson’s correlation was used to determine which responses and which genetic polymorphisms were related to days on treatment. Stepwise multiple regression was used to determine the model for each outcome considering the four genetic polymorphisms, days on LG839 treatment and extent to which subjects followed instructions. R² correlations were given for the final model’s ability to explain variance in the outcome measurement. These were contribution to the overall variance of a pre-assigned total sum outcome index (weight loss in kg, loss of waist circumference in inches, sugar cravings, snack intake, late night binging behavior, exercise, appetite, and energy level).

In addition, Cronbach’s alpha was used to determine that the index sum indeed was reliable. It was reliable, as Cronbach’s alpha was 0.73 for the total sum of change scores. This total provides an over all response to use for the multiple regression, in addition to the individual item outcomes measured in the questionnaire.

Percentages of each gene polymorphism for the n=1,058 self identified sample of obese and for the variable sample size of normal controls from the literature were each compared to the sample of n=27 in this study for each gene polymorphism individually by Fisher’s exact test. We also pooled the genotypes from each group (1058 obese subjects; 27 obese subjects [subset]; literature controls) and statistically compared genotypic patterns for significant differences.

### III. Results

Even with this small sample size, we obtained significant polymorphic correlates with regard to a number of well-known genes (PPAR γ2, MTHFR, 5-HT2a, and DRD2 genes). In this regard, carrying certain known polymorphisms (MTHFR - C677T allele; OB - D7S1875 allele; 5-HT2a - 1438G/A allele; DRD2 – A1 allele; PPAR- γ2 - Pro12Ala allele) correlated negatively with positive clinical parameters tested in this study. The percentage prevalence for each gene polymorphism is presented in table 3. In order to determine weather a genetic difference occurred between the 1058 Dutch subjects participating in the overall D.I.E.T. study and the subtype participants (n=27) utilized in the present study a Chi Square test revealed no difference between any gene polymorphism evaluated for those present in each polymorphism compared to the average for all three groups. There were some missing data and thus a n=23 was a mean for all genes tested. Some gene polymorphisms differed among the three groups, but the sample of n=23 always had a percentage near the average for all three groups and thus did not differ from the overall gene polymorphism distribution. These percentages were 31.3% overall for the DRD2 gene and 39.1% in the sample here. The MTHFR gene was present 68.4% overall and 64.0% in this sample. The 5-HT2a receptor gene was present 63.5% overall and 64.0% in this sample. The PPAR-γ gene was present 17.5% overall and 17.4% for this sample (Table 3). Due to missing data instead of n=27 the various means were calculated using n=23.

In particular for this study of all the outcomes and gene polymorphisms, only the DRD2 gene polymorphism had a significant Pearson correlation with days on treatment (τ=0.42, p=0.045) (Figure 2). In this regard, compared to the DRD2 A carriers the number of days in treatment with LG839 was 51.9 ± 9.9 SE (95% CI, 30.8 to 73.0 ) and for the DRD2 A carrier the number of days on treatment with LG839 was 110.6 ± 31.1 (95% CI, 38.9 to 182.3 ). Since the standard errors were different some frequency data was also computed. The proportion of DRD2 A+ subjects with greater than 50 days on treatment was 5/14 (36%) and the proportion of DRD2 A+ with greater than 50 days on treatment was 7/9 (78%) with chi-square p-value of 0.049. Both of these tests are near 0.05 but both have mean differences of the two groups that reflect a robust two-fold differential outcome in terms of days on treatment with LG839.

### Table 3. Genotypic comparisons between the 1058 obesity sample, the 27 subset of obese subjects, and literature controls

<table>
<thead>
<tr>
<th>Gene Studied</th>
<th>Percent Prevalence</th>
<th>Percent Prevalence</th>
<th>Percent Prevalence</th>
<th>Frequency For [Wild Type]</th>
<th>Frequency For [Mutant Type]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Name &amp; Description</td>
<td>Mutant (combined</td>
<td>Obesity sample</td>
<td>Literature control</td>
<td>Wild type Sample</td>
<td>Mutant type Sample</td>
</tr>
<tr>
<td></td>
<td>Heterozygous/Homozygous</td>
<td></td>
<td>Sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat regulator (PPAR)</td>
<td>4/23 (17.4)</td>
<td>265/1058 (25.1)</td>
<td>314/2244 (14.0)</td>
<td>0.90</td>
<td>0.10</td>
</tr>
<tr>
<td>Nervous eating (5-H</td>
<td>16/25 (64.0)</td>
<td>679/1058 (64.2)</td>
<td>173/283 (61.1)</td>
<td>0.56</td>
<td>0.44</td>
</tr>
<tr>
<td>HT2a)</td>
<td></td>
<td></td>
<td>54/100 (54.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New cell (MTHFR)</td>
<td>16/25 (64.0)</td>
<td>739/1058 (69.9)</td>
<td>945/3258 (29.0)</td>
<td>0.76</td>
<td>0.24</td>
</tr>
<tr>
<td>Sweet tooth (DRD2)</td>
<td>9/23 (39.1)</td>
<td>403/1058 (38.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Represents the significant differences (P< 0.045) between the DRD2 A1- (n= 9) and the DRD2 A1+ (n= 14) with regard to days on LG839 treatment in Dutch descent self-identified obese subjects in the D.I.E.T. pilot study. A1+ = (A1/A1/A1/A2) and A1- = A2/A2.

In an attempt to determine the contribution of each polymorphism for the four genes tested with regard to LG839 induced outcomes, we utilized a simple t-test for such comparisons. When we compared the DRD2 A1- with the DRD2 A1+ we found no significant differences in any outcome parameters tested other than the days on treatment.

IV. Discussion

Obesity is a biogenetic condition involving both environmental and genetic antecedents. Like certain health conditions that were once classified as purely behavioral abnormalities, obesity is now accurately characterized as a disease with biological and genetic factors. It has been reported that in both Caucasian women and men, heritability for BMI was 0.58 and 0.63, for body fat %, 0.59, and 0.63, and for lean body mass 0.61 and 0.56, respectively (Males et al, 1997). Because of the complexity of obesity and related conditions involving reward deficiencies, it is likely that more than one defective gene is involved. However, work from Volkow's lab showed a dopamine D2 receptor paucity in subjects with Reward Deficiency Syndrome (RDS) [defined as obese individuals] vs. non-RDS subjects [defined as non obese individuals] by PET scan (Wang et al, 2001, 2004).

This is the first study that we are aware of to evaluate genetics and treatment compliance concerning a nutraceutical as an anti-obesity agent. Medication noncompliance is a significant concern for various health conditions, including weight-related treatments. One half of the patients for whom appropriate medication is prescribed fail to receive the full benefits because of inadequate adherence to treatment (Meichenbaum and Turk, 1987). It is interesting to note in another study 77% of patients complied with their medication when it was designed to cure a disease versus only 63% of patients complied when treatment was aimed at prevention. However, when taken over a long period, medication compliance rates dropped dramatically to approximately 50% for either prevention or cure (Buckalew and Sallos, 1986). Some studies show that 20% to 80% of patients make errors in taking medication and that 20% to 60% stop taking medications before being instructed to do so (Meichenbaum and Turk, 1987; Sackett and Snow, 1979). Within the elderly population which tend to more likely receive medication, the literature concerning adherence reports that compliance rates range roughly from 38% to 57%, with an average rate of less than 45% (Dunbar, 1979; Sackett and Snow, 1979). According to the American Heart Association (AHA), in 2007, medication noncompliance is a serious health threat. They state that the number one problem in treating illness today is patients’ failure to take prescription medications correctly. They go onto explain that almost 29 percent of Americans stop taking their medicine before it runs out, 22 percent of Americans take less of the medication than is prescribed on the label, 12 percent of Americans don't fill their prescription at all, and 12 percent of Americans don't take medication at all after they buy the prescription. This non compliance results in huge costs. According to the AHA, 10 percent of all hospital admissions are the result of patients failing to take prescription medications correctly which result in an average hospital stay of 4.2 days and 23 percent of all nursing home admissions are due to patients failing to take prescription medications accurately.

Some have estimated that the cost of this noncompliance exceeds $270 million daily in additional hospitalizations and other medical costs (Doctors Release, 2007). In this study, albeit small sample size, we have demonstrated that the DRD2 genotype may associate with treatment compliance on LG839, and possibly should be explored as a genotypic marker for treatment compliance of other therapies, including prescription medications.
While the subset of 27 subjects [mean data derived from n=23] may seem low in terms of response, it is within the expected percentage of individuals that respond to any direct marketing communication. In fact it is well known that the rate of response is between 1 and 3%, and in light of subjects not receiving any incentive or compensation for their response, this was an adequate response. Additionally, statistics obtained from the Society of Consumer Affairs Professionals (SOCAP) in Business shed further light on the nature of this type of data.

One would expect that when consumers have the choice to communicate with an entity, it is generally to complain and not compliment. Thus, this data would have a negative bias, in that consumers would have a reason to report negative results over positive results. From this analysis of 27 respondents, 23 out of 27 (85 percent) communicated email and phone complaints (e.g. slow delivery of product etc) to the Company related to the customer service of the local Netherlands call center. This may introduce a negative bias against the intervention in this study, reducing any perceived positive outcomes. In data obtained from SOCAP, it is noted that the 59 percent of such communications via phone and email were generally information requests, and the second leading category across both communication mediums was Complaints/Negative Opinions (29 percent). This data confirms the negative bias of the survey respondents (Personal communication - Cindy Collins Smith Publications Coordinator, CRM Editor SOCAP International, Alexandria, Virginia).

Albeit a small sample size, it is very interesting that of all the genes tested a positive correlation was obtained with the DRD2 A1 allele and number of days in treatment. This takes on significance because it would appear that those individuals carrying the DRD2 A1 allelic polymorphism continued treatment due to potential benefits of LG839 targeted at a deficiency of dopamine receptor (D2) density. In this regard, similar findings (Lawford et al, 1995) have been reported for the drug bromocriptine, a dopamine D2 receptor agonist, in the treatment of alcoholics with the D2 dopamine receptor A1 allele. In a double-blind study, bromocriptine, or placebo was administered to alcoholics with either A1 (A1/A1 and A1/A2 genotypes) or only A2 (A2/A2 genotypes) allele of the DRD2 gene. In the Lawford and colleagues study, the greatest improvement in craving and anxiety occurred in the bromocriptine-treated A1 alcoholics and attrition (number of days in treatment) was highest in the placebo-treated A1 alcoholics. It is possible that one important benefit of LG839 is its ability to reduce sugar craving (Blum et al, 2006, 2007b) and increase energy (Epstein et al, 2007).

A Ser311Cys mutation of the human DRD2 produces a marked functional impairment of the receptor and is associated with higher BMI in some populations. Tataranni and colleagues hypothesized in 2001 that the Ser311Cys mutation of DRD2 may inhibit energy expenditure. They have reported that total energy expenditure (doubly labeled water) measured in 89 non-diabetic Pima Indians was 244 kcal/ day lower in homozygotes for the Cys311-encoding allele when compared with those heterozygous and homozygous for the Ser311-encoding allele (P = 0.056). The 24-h resting energy expenditure (respiratory chamber) measured in 320 nondiabetic Pimas was also 87 kcal/day lower in homozygotes for the Cys311-encoding allele when compared with those heterozygous and homozygous for the Ser311-encoding allele (p=0.026). These findings were the first evidence that a genetic mutation is associated with reduced energy expenditure in humans.

It is noteworthy that since we found no difference in LG839 induced outcomes, we carefully suggest that the driving force behind compliance (2 fold) in this population studied was the presence of the DRD2 A1+ genotype rather than benefit outcomes. This may suggest that we have potentially found a useful persistence genotype (Lawford et al, 1995) that may have importance pharmacogenetically across therapeutic modalities (pharmaceuticals, nutraceuticals and medical foods) concerning RDS behaviors.

Due to the small sample size of 14 negative and 9 positive for DRD2 A1 allele, the results are very sensitive and larger sample size is needed. Further studies with a larger number of obese subjects in a randomized double-blinded, placebo-controlled study is warranted before any firm conclusions could be drawn from this data set.

V. Conclusion
In conclusion, obesity, a heterogeneous disease with hereditary and environmental determinants, is a major health and social problem with a high recidivism rate. With the recent FDA rejection of the CB1 receptor blocking agent Acomplia™ a continued search for novel anti-obesity agents is of utmost importance. The advent of molecular genetic techniques makes possible the identification of genetic types of obese individuals. Such identification renders feasible specific pharmacogenetic and pharmacogenomic approaches to the treatment of hereditary forms of obesity. This experiment on the DRD2 gene and LG839 describes one such approach. However any interpretation must await further double-blinded large sample size studies confirming these potentially interesting results.

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References


