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## RESEARCH: PHARMACOGENOMICS

# GENETIC ADDICTION RISK SCORE (GARS) ANALYSIS: EXPLORATORY DEVELOPMENT OF POLYMORPHIC RISK ALLELES IN POLY-DRUG ADDICTED MALES

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## ABSTRACT

*There is a need to classify patients at genetic risk for drug seeking behavior prior to or upon entry to residential and or non-residential chemical dependency programs. We have determined based on a literature review, that there are seven risk alleles associated with six candidate genes that were studied in this patient population of recovering poly-drug abusers. To determine risk severity of these 26 patients we calculated the percentage of prevalence of the risk alleles and provided an arbitrary severity score based on percentage of these alleles. Subjects carry the following risk alleles: DRD2=A1; SLC6A3 (DAT) =10R; DRD4=3R or 7R; 5HTTIRP = L or LA; MAO= 3R; and COMT=G. As depicted in table 2 low severity (LS) = 1-36%; Moderate Severity =37-50%, and High severity = 51-100%. We studied two distinct ethnic populations group 1 consisted of 16 male Caucasian psycho stimulant addicts and group 2 consisted of 10 Chinese heroin addicted males. Based on this model the 16 subjects tested have at least one risk allele or 100%. Out of the 16 subjects we found 50% (8) HS; 31% (5) MS; and 19% LS (3 subjects). These scores are then converted to a fraction and then represented as a Genetic Addiction Risk Score (GARS) whereby we found the average GARS to be: 0.28 low severity, 0.44 moderate severity and 0.58 high severity respectively. Therefore, using this GARS we found that 81 % of the patients were at moderate to high risk for addictive behavior. Of particular interest we found that 56 % of the subjects carried the DRD2 A1 allele (9/16). Out of the 9 Chinese heroin addicts [one patient not genotyped] (group 2) we found 11% (1) HS; 56% (5) MS; and 33% LS (3 subjects). These scores are then converted to a fraction and then represented as GARS whereby we found the average GARS to be: 0.28 Low Severity; 0.43 moderate severity and 0.54 high severity respectively. Therefore, using GARS we found that 67 % of the patients were at moderate to high risk for addictive behavior. Of particular interest we found that 56 % of the subjects carried the DRD2 A1 allele (5/9) similar to group 1. Statistical analysis revealed*

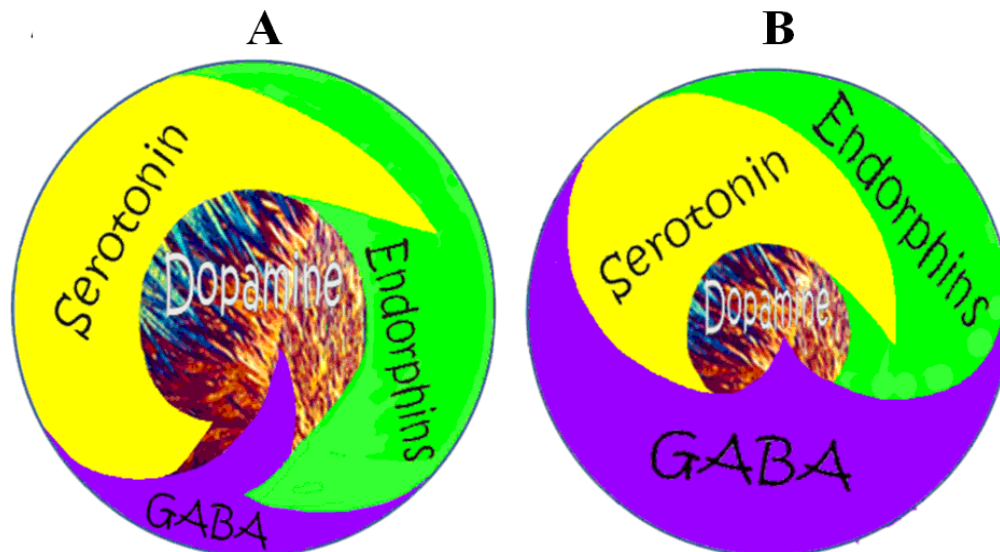
that the groups did not differ in terms of overall severity (67 vs. 81%) in these two distinct populations. Combining these two independent study populations reveal that subjects entering a residential treatment facility for poly-drug abuse carry at least one risk allele (100%). We found 74% of the combined 25 subjects (Caucasian and Chinese) had a moderate to high GARS. Confirmation of these exploratory results and development of mathematical predictive values of these risk alleles are necessary before any meaningful interpretation of these results are to be considered.

**Keywords:** Genetic Addiction Risk Score (GARS); polymorphic genes; Neurotransmitters; Dopamine; Reward Deficiency Syndrome (RDS)

## [I] INTRODUCTION

Over half a century of dedicated and rigorous scientific research on the meso-limbic system provided insight into the addictive brain and the neurogenetic mechanisms involved in man's quest for happiness. In brief, the site of the brain where one experiences feelings of well being is the meso-limbic system. This part of the brain has been termed the "reward center". Chemical messages including serotonin, enkephalins, GABA and dopamine (DA), work in concert to provide a net release of DA at the nucleus accumbens (NAc), a region in the mesolimbic system. It is well known that genes control the synthesis,

vesicular storage, metabolism, receptor formation and neurotransmitter catabolism. The polymorphic-versions of these genes have certain variations which could lead to an impairment of the neurochemical events involved in the neuronal release of DA. The cascade of these neuronal events has been termed "Brain Reward Cascade" [1] [Figure-1]. A breakdown of this cascade will ultimately lead to a dysregulation and dysfunction of DA. Since DA has been established as the "pleasure molecule" and the "anti-stress molecule," any reduction in function could lead to reward deficiency and resultant aberrant substance seeking behavior and a lack of wellness [2].



**Fig: 1. Brain Reward Cascade.** (A) Schematic represents the normal physiologic state of the neurotransmitter interaction at the mesolimbic region of the brain. Briefly in terms of the "Brain Reward Cascade" first coined by Blum and Kozlowski [90]: serotonin in the hypothalamus stimulates neuronal projections of methionine enkephalin in the hypothalamus which in turn inhibits the release of GABA in the substantia nigra thereby allowing for the normal amount of Dopamine to be released at the NAc (reward site of Brain). (B) Represents hypodopaminergic function of the mesolimbic region of the brain. It is possible that the hypodopaminergic state is due to gene polymorphisms as well as environmental elements including both stress and neurotoxicity from aberrant abuse of psychoactive drugs (*i.e. alcohol, heroin, cocaine etc*). Genetic variables could include serotonergic genes (serotonergic receptors [5HT<sub>2a</sub>]; serotonin transporter 5HT<sub>1PR</sub>); endorphinergic genes (mu OPRM1 gene; proenkephalin (PENK) [PENK polymorphic 3' UTR dinucleotide (CA) repeats]; GABergic gene (GABRB3) and dopaminergic genes (ANKK1 Taq A; DRD2 C957T, DRD4 7R, COMT Val/met substitution, MAO-A uVNTR, and SLC6A3 9 or 10R). Any of these genetic and or environmental impairments could result in reduced release of dopamine and or reduced number of dopaminergic receptors.

*Homo sapiens* are biologically predisposed to drink, eat, reproduce and desire pleasurable experiences. Impairment in the mechanisms involved in these natural processes lead to multiple impulsive, compulsive and addictive behaviors governed by genetic polymorphic antecedents. While there are a plethora of genetic variations at the level of mesolimbic activity, polymorphisms of the serotonergic- 2A receptor (5-HTT2a); serotonergic transporter (5HTTLPR); (dopamine D2 receptor (DRD2), Dopamine D4 receptor (DRD4) ; Dopamine transporter (DAT1); and the Catechol-o-methyl –transferase (COMT) , monoamine –oxidase (MOA) genes as well as other candidate genes predispose individuals to excessive cravings and resultant aberrant behaviors [3].

An umbrella term to describe the common genetic antecedents of multiple impulsive, compulsive and addictive behaviors is Reward Deficiency Syndrome (RDS). Individuals possessing a paucity of serotonergic and/or dopaminergic receptors and an increased rate of synaptic DA catabolism, due to high catabolic genotype of the COMT gene, or high MOA activity are predisposed to self-medicating with any substance or behavior that will activate DA release including alcohol, opiates, psychostimulants, nicotine, glucose, gambling, sex, and even excessive internet gaming, among others [4]. Use of most drugs of abuse, including alcohol, is associated with release of dopamine in the mesocorticolimbic system or “reward pathway of the brain [5]. Activation of this dopaminergic system induces feelings of reward and pleasure [6, 7]. However, reduced activity of the dopamine system (hypodopaminergic functioning) can trigger drug-seeking behavior [8, 9]. Variant alleles can induce hypodopaminergic functioning through reduced dopamine receptor density, blunted response to dopamine, or enhanced dopamine catabolism in the reward pathway [10]. Possibly, cessation of chronic drug use induces a hypodopaminergic state that prompts drug-seeking behavior in an attempt to address the withdrawal –induced state [11].

Acute utilization of these substances can induce a feeling of well being. But, unfortunately sustained and prolonged abuse leads to a toxic pseudo feeling of well being resulting in tolerance and dis-ease or discomfort. Thus, low DA receptors due to carrying the DRD2 A1 allelic genotype results in excessive cravings and consequential behavior, whereas normal or high DA receptors results in low craving induced behavior. In terms of preventing substance abuse, or excessive glucose craving, one goal would be to induce a proliferation of DA D2 receptors in genetically prone individuals [12]. Experiments in vitro have shown that constant stimulation of the DA receptor system via a known D2 agonist in low doses results in significant proliferation of D2 receptors in spite of genetic antecedents [13]. In essence, D2 receptor stimulation signals negative feedback mechanisms in the mesolimbic system to induce mRNA expression causing proliferation of D2 receptors. This molecular finding serves as the basis to naturally induce DA release to also cause the same induction of D2-directed mRNA and thus proliferation of D2 receptors in the human.

This proliferation of D2 receptors in turn, will induce the attenuation of craving behavior. In fact this has been proven with work showing DNA–directed over-expression (a form of gene therapy) of the DRD2 receptors and significant reduction in both alcohol and cocaine craving-induced behavior in animals [14, 15].

These observations are the basis for the development of a functional hypothesis of drug –seeking and drug use. The hypothesis is that the presence of a hypodopaminergic state, regardless of the source, is a primary cause of drug –seeking behavior. Thus, genetic polymorphisms that induce hypodopaminergic functioning may be the causal mechanism of a genetic predisposition to chronic drug use and relapse [12]. Finally, utilizing the long term dopaminergic activation approach will ultimately lead to a common safe and effective modality to treat RDS behaviors including Substance Use Disorders (SUD), Attention Deficit Hyperactivity Disorder (ADHD), and Obesity among other reward deficient aberrant behaviors.

Support for the impulsive nature of individuals possessing dopaminergic gene variants is derived from a number of important studies illustrating the genetic risk for drug-seeking behaviors based on association and linkage studies implicating these alleles as risk antecedents having impact in the mesocorticolimbic system. REF

### 1.1 D2 dopamine receptor gene (DRD2)

The dopamine D2 receptor gene (DRD2) first associated by Blum *et al* [17] with severe alcoholism is the most widely studied candidate gene in psychiatric genetics. The *Taq1 A* is a single nucleotide polymorphism (SNP rs: 1800497) originally thought to be located at the 3' untranslated region of the DRD2 but now has been shown to be located within exon 8 of an adjacent gene, the ankyrin repeat and kinase domain containing 1 ( ANKK1) [18]. Importantly, while there may be distinct differences in function, Neville *et al* [18] suggest that the miss-location of the *Taq1 A* may be attributable to the ANKKI and the DRD2 being on the same haplotype or the ANKKI being involved in reward processing through a signal transduction pathway. The ANKKI and the DRD2 gene polymorphisms may have distinct different actions with regard to brain function as has been noted in recent experiments and fear related conditioning in alcoholics [19, 20]. Grandy *et al.* [21] reported on the presence of the two alleles of the *Taq1 A*: the A1 and A2 . Presence of the A1<sup>+</sup> genotype (A1/A1, A1 /A2) compared to the A<sup>-</sup> genotype (A2/A2), is associated with reduced density [22, 23]. This reduction causes hypodopaminergic functioning in the dopamine reward pathway. Noble [24] in reviewing the literature concluded that research supports a predictive relationship from the A1<sup>+</sup> genotype to drug seeking behavior. This has been also discussed by Blum *et al* [3, 25] reporting that presence of the A+ genotype using Bayesian analysis has a predictive value of 74% for a number of RDS behaviors.

Other DRD2 polymorphisms such as the C (57T, A SNP (rs: 6277) at exon 7 also associates with a number of RDS behaviors including drug use [26, 27, 28]. Compared to the T<sup>-</sup> genotype (C/C), the T<sup>+</sup> genotype (T/T, T/C) is associated with reduced translation of DRD2 mRNA and diminished DRD2 mRNA [26], leading to reduced DRD2 density [27]. Hill *et al.* [28] has shown the predictive relationship between the T<sup>+</sup> allele and alcohol dependence. This results in hypodopaminergic function and is also a predictive risk allele.

The association of the DRD2 A1 allele in alcoholism is well established showing in a 10 year follow up that carriers of the DRD2 A1 allele have a higher rate of mortality compared to carriers of the A2 allele in alcohol dependent individuals [29]. There are 390 PUBMED reports [6/5/2010] providing significant support. The dopamine D2 receptor (DRD2) plays an important role in the reinforcing and motivating effects of ethanol. Several polymorphisms have been reported to effect receptor expression. The amount of DRD2, expressed in a given individual, is the result of the expression of both alleles, each representing a distinct haplotype.

Most recently, Kraschewski *et al.* [30] found that the haplotypes I-C-G-A2 and I-C-A-A1 occurred with a higher frequency in alcoholics [P=0.026, odds ratio (OR): 1.340; P=0.010, OR: 1.521, respectively]. The rare haplotype I-C-A-A2 occurred less often in alcoholics (P=0.010, OR: 0.507), and was also less often transmitted from parents to their affected offspring (1 vs.7). Among the subgroups, I-C-G-A2 and I-C-A-A1 had a higher frequency in Cloninger 1 alcoholics (P=0.083 and 0.001, OR: 1.917, respectively) and in alcoholics with a positive family history (P=0.031, OR: 1.478; P=0.073, respectively). Cloninger 2 alcoholics had a higher frequency of the rare haplotype D-T-A-A2 (P<0.001, OR: 4.614) always compared with controls. In patients with positive family history haplotype I-C-A-A2 (P=0.004, OR: 0.209), and in Cloninger 1 alcoholics haplotype I-T-A-A1 (P=0.045 OR: 0.460) were less often present. They confirmed the hypothesis that haplotypes, which are supposed to induce a low DRD2 expression, are associated with alcohol dependence. Furthermore, supposedly high-expressing haplotypes weakened or neutralized the action of low-expressing haplotypes.

## 1.2 D4 dopamine receptor gene (DRD4)

There is evidence that the length of the D4 dopamine receptor (DRD4) exon 3 variable number of tandem repeats (VNTR) affects DRD4 functioning by modulating the expression and efficiency of maturation of the receptor [31]. The 7 repeat (7R) VNTR requires significantly higher amounts of dopamine to produce a response of the same magnitude as other size VNTRs [32]. This reduced sensitivity or “dopamine resistance” leads to hypodopaminergic functioning. Thus 7R VNTR has been associated with substance-seeking behavior [32, 33]. However not all reports support this association [34]. Most recently Biederman *et al.* [35] evaluated a number of putative risk alleles

using survival analysis, revealed that by 25 years of age 76% of subjects with a DRD4 7-repeat allele were estimated to have significantly more persistent ADHD compared with 66% of subjects without the risk allele. In contrast, there were no significant associations between the course of ADHD and the DAT1 10-repeat allele (P=0.94) and 5HTTLPR long allele. Their findings suggest that the DRD4 7-repeat allele is associated with a more persistent course of ADHD. This is consistent with our finding of the presence of the 7R DAT genotype in the heroin addict. Moreover in a study by Grzywacz *et al.* [36] which evaluated the role of dopamine D4 receptor (DRD4) exon 3 polymorphisms (48 bp VNTR) in the pathogenesis of alcoholism, they found significant differences in the short alleles (2-5 VNTR) frequencies between controls and patients with a history of delirium tremens and/or alcohol seizures (p = 0.043). A trend was also observed in the higher frequency of short alleles amongst individuals with an early age of onset of alcoholism (p = 0.063). The results of this study suggest that inherited short variants of DRD4 alleles (3R) may play a role in pathogenesis of alcohol dependence and carriers of the 4R may have a protective effect for alcoholism risk behaviors. It is of further interest that work from Kotler *et al.* [37] in heroin addicts illustrated that central dopaminergic pathways figure prominently in drug-mediated reinforcement including novelty seeking, suggesting that dopamine receptors are likely candidates for association with substance abuse in man. These researchers show that the 7-repeat allele is significantly over-represented in the opioid-dependent cohort and confers a relative risk of 2.46.

## 1.3 Dopamine Transporter gene (DAT1)

The dopamine transporter protein regulates dopamine-mediated neurotransmission by rapidly accumulating dopamine that has been released into the synapse [38]. The dopamine transporter gene (SLC6A3 or DAT1) is localized to chromosome 5p15.3. Moreover, within 3 non-coding region of DAT1 lies a VNTR polymorphism [38]. There are two important alleles that may independently increase risk for RDS behaviors. The 9 repeat (9R) VNTR has been shown to influence gene expression and to augment transcription of the dopamine transporter protein [39]. Therefore this results in an enhanced clearance of synaptic dopamine, yielding reduced levels of dopamine to activate postsynaptic neurons. Presence of the 9R VNTR has been linked to Substance Use Disorder (S.U.D.) [40] not consistently [41]. Moreover in terms of RDS behaviors, Cook *et al.* [42] was the first group that associated tandem repeats of the dopamine transporter gene (DAT) in the literature. While there have been some inconsistencies associated with the earlier results the evidence is mounting in favor of the view that the 10R allele of DAT is associated with high risk for ADHD in children and in adults alike. Specifically, Lee *et al.* [43] found consistent support in several studies, the non-additive association for the 10-repeat allele was significant for hyperactivity-impulsivity (HI) symptoms. However, consistent with other studies, exploratory analyses of the non-additive association of the 9-

repeat allele of DAT1 with HI and oppositional defiant disorder (ODD) symptoms also were significant.

#### 1.4 Catechol-O-methyltransferase (COMT)

The catechol-O-methyltransferase (COMT) is an enzyme involved in the metabolism of dopamine, adrenaline and noradrenaline. The Val158Met polymorphism of the COMT gene has been previously associated with a variability of the COMT activity, and alcoholism. Serý [44] found a relationship between the Val158Met polymorphism of the COMT gene and alcoholism in male subjects. Serý [44] found the significant difference between male alcoholics and male controls in allele and genotype frequencies ( $p < 0.007$ ; and  $p < 0.04$  respectively). Interestingly in one of the subjects genotyped herein who battles with heroin as an addiction while carrying the DRD2 A1 allele also carried the low enzyme COMT activity genotype (A/A). This is agreement with the work of Cao *et al.* [45] who did not find an association with the high G/G and heroin addiction. No differences in genotype and allele frequencies of 108 val/met polymorphism of COMT gene were observed between heroin-dependent subjects and normal controls (genotype-wise: chi-square=1.67,  $P=0.43$ ; allele-wise: chi-square=1.23,  $P=0.27$ ). No differences in genotype and allele frequencies of 900 Ins C/Del C polymorphism of COMT gene were observed between heroin-dependent subjects and normal controls (genotype-wise: chi-square=3.73,  $P=0.16$ ; allele-wise: chi-square=0.76,  $P=0.38$ ). While there is still some controversy regarding the COMT association with heroin addiction it was also interesting that the A allele of the val/met polymorphisms (-287 A/G) found by Cao *et al.* [45] was found to be much higher in heroin addicts than controls. Faster metabolism results in reduced dopamine availability at the synapse, which reduces postsynaptic activation, inducing hypodopaminergic functioning. Generally Vanderbergh *et al.* [46] and Wang *et al.* [47] support an association with the Val allele and SUD but others do not [48].

#### 1.5 Monoamine -Oxidase A

Monoamine oxidase-A (MAOA) is a mitochondrial enzyme that degrades the neurotransmitters serotonin, norepinephrine, and dopamine. This system is involved with both psychological and physical functioning. The gene that encodes MAOA is found on the X chromosome and contains a polymorphism (MAOA-uVNTR) located 1.2 kb upstream of the MAOA coding sequences [49]. In this polymorphism, consisting of a 30-base pair repeated sequence, six allele variants containing either 2-, 3-, 3.5-, 4-, 5-, or 6-repeat copies have been identified [50]. Functional studies indicate that certain alleles may confer lower transcriptional efficiency than others. The 3-repeat variant conveys lower efficiency, whereas 3.5- and 4-repeat alleles result in higher efficiency [51]. The 3- and 4-repeat alleles are the most common, and to date there is fewer consensus regarding the transcriptional efficiency

of the other less commonly occurring alleles (e.g., 2-, 5-, and 6-repeat). The primary role of MAOA in regulating monoamine turnover, and hence ultimately influencing levels of norepinephrine, dopamine, and serotonin, indicates that its gene is a highly plausible candidate for affecting individual differences in the manifestation of psychological traits and psychiatric disorders [52]. For example, recent evidence indicates that the MAOA gene may be associated with depression [53] and stress [54]. However, evidence regarding whether higher or lower MAOA gene transcriptional efficiency is positively associated with psychological pathology as been mixed. The low-activity 3-repeat allele of the MAOA-uVNTR polymorphism has been positively related to symptoms of antisocial personality [55] and cluster B personality disorders. Other studies, however, suggest that alleles associated with higher transcriptional efficiency are related to unhealthy psychological characteristics such as trait aggressiveness and impulsivity. Low MAO activity and the neurotransmitter dopamine are 2 important factors in the development of alcohol dependence. MAO is an important enzyme associated with the metabolism of biogenic amines. Therefore, Huang *et al.* [56] investigated whether the association between the dopamine D2 receptor (DRD2) gene and alcoholism is affected by different polymorphisms of the MAO type A (MAOA) gene. The genetic variant of the DRD2 gene was only associated with the anxiety, depression (ANX/DEP) ALC phenotype, and the genetic variant of the MAOA gene was associated with ALC. Subjects carrying the MAOA 3-repeat allele and genotype A1/A1 of the DRD2 were 3.48 times (95% confidence interval = 1.47-8.25) more likely to be ANX/DEP ALC than the subjects carrying the MAOA 3-repeat allele and DRD2 A2/A2 genotype. The MAOA gene may modify the association between the DRD2 gene and ANX/DEP ALC phenotype. Overall, Vanyukov *et al.* suggested that, although not definitive, variants in MAOA account for a small portion of the variance of SUD risk, possibly mediated by liability to early onset behavioral problems [57].

#### 1.6 Serotonin Transporter gene

The human serotonin (5-hydroxytryptamine) transporter, encoded by the SLC6A4 gene on chromosome 17q11.1-q12, is the cellular reuptake site for serotonin and a site of action for several drugs with central nervous system effects, including both therapeutic agents (e.g. antidepressants) and drugs of abuse (e.g. cocaine). It is known that the serotonin transporter plays an important role in the metabolic cycle of a broad range of antidepressants, antipsychotics, anxiolytics, antiemetics, and anti-migraine drugs. Salz *et al.* [58] found an excess of -1438G and 5-HTTLPR L carriers in alcoholic patients in comparison to the heroin dependent group (OR (95% CI)=1.98 (1.13-3.45) and 1.92 (1.07-3.44), respectively). The A-1438G and 5-HTTLPR polymorphisms also interacted in distinguishing alcohol from heroin dependent patients ( $df = 10.21$  (4),  $p=0.037$ ). The association of -1438A/G with alcohol dependence was especially pronounced in the presence of 5-HTTLPR S/S, less evident with 5-HTTLPR L/S and not present with 5-HTTLPR

L/L. SCL6A4 polymorphism haplotypes were similarly distributed in all three groups. Moreover, Seneviratne *et al.* [59] found that G allele carriers for rs1042173 were associated with significantly lower drinking intensity ( $p = 0.0034$ ) compared to T-allele homozygotes. In HeLa cell cultures, the cells transfected with G allele showed a significantly higher mRNA and protein levels than the T allele-transfected cells. These findings suggest that the allelic variations of rs1042173 affect drinking intensity in alcoholics possibly by altering serotonin transporter expression levels. This provides additional support to the hypothesis that SLC6A4 polymorphisms play an important role in regulating propensity for severe drinking.

### 1.7 Combination of Genes and Addiction Risk

In general inconsistencies in the literature involving association studies using single gene analysis prompted Conner *et al.* [60] and others to evaluate a number of dopaminergic gene polymorphisms as predictors of drug use in adolescents. We can't ignore the importance of neurochemical mechanisms involved in drug induced relapse behavior as suggested by Bossert *et al.* [61] understanding the interaction of multiple genes and environmental elements. These investigators have found using a drug relapse model, previously shown to induce relapse by re-exposing rats to heroin-associated contexts. After extinction of drug-reinforced responding in different contexts, re-exposure reinstates heroin seeking. This effect is attenuated by inhibition of glutamate transmission in the ventral tegmental area and medial accumbens shell, components of the mesolimbic dopamine system. This process enhances DA net release in the NAc. This fits well with Li's KARG addiction network map [62].

Since the initial finding of Blum *et al.* [17] showing positive association of a single gene DRD2 polymorphisms and severe alcoholism to date the replication, although favorable, has been fraught with inconsistent results. This has been true for other complex behaviors as well (NCI-NHGRI Working Group on Replication in Association studies 2007). Moreover, when gene-gene and environment interactions are tested the findings support the concept that complex gene –relationships may account for inconsistent findings across many different single gene studies [63].

There are many different reasons for inconsistencies in trying to predict drug use including single gene analysis, stratification of population, poor screened controls, gender–base differences, personality traits, co-morbidity of psychiatric disorders, positive and negative life events and even neurocognitive functioning [64, 65].

Thus, instead of continuing to evaluate single gene associations to predict future drug abuse, it occurred to us that we should embark on a study to evaluate multiple candidate gene candidates especially linked to the Brain Reward Cascade and

hypodopaminergic functioning to gain a more complex but stronger predictive set of genetic antecedents. Our goal albeit exploratory in nature is to develop an informative panel to provide a means of stratifying or classifying patients entering a treatment facility as having low, moderate or high genetic predictive risk based on a number of known risk alleles. We are coining the term **Genetic Addiction Risk Score (GARS)** for purposes of study identification.

## [II] MATERIALS AND METHODS

### 2.1 Subjects

The genotype data utilized in this paper is derived from previously published papers concerned with qEEG response from a natural Dopamine D2 agonist called Synaptose™ [64, 65] but the data set was never combined as accomplished herein.. The 16 patients were interviewed and evaluated for chemical dependence using a standard battery of diagnostic tests and questionnaires. The tests included the following: *Drug History Questionnaire; Physical Assessment, Urine Drug Tests; breathalyzer; Complete CBC blood test; and Symptom Severity Questionnaire.* The patients were determined to be substance dependent according to *Diagnostic and Statistical Manual [DSM-IV]* criteria. All patients were residential patients at G & G Holistic Addiction Treatment Center, North Miami Beach, Florida [14 patients] and the Bridging the Gaps, Winchester, Virginia [2 patients] treatment programs (30-90 day chemical dependence rehabilitation program). All subjects signed an approved consent form (approved by the IRB at PATH Foundation NY, New York, New York, registration # IRB00002334) and agreed to volunteer for this feasibility study. For protection of the patients the genotyping data conformed to standard HIPPA and GINA practices mandated by law.

**Table-1** shows the demographics of the overall population including gender, race, age and length of abstinence. In this study there were a total of sixteen individuals. There were 16 Males and 0 females with a median age of  $29.5 \pm 8.8$  SD years. The population breakdown was as follows: 87.5 % Caucasian, and 12.5 Percent Hispanic. The average number of months abstinent for the entire population was  $9.5 \pm 23.3$ . There were 3 pure cocaine only addicts; 4 cocaine crack addicts; 9 cocaine plus other drugs of abuse (alcohol, opiates and marijuana).

**Table 1. Demographics of all Caucasian subjects combined**

	Median $\pm$ st.dev.	(min, max)	N (total = 16)
Age	29.5 $\pm$ 8.80	(19, 48)	16
Clean time	9.5 $\pm$ 23.33	(2, 101)	16
Race =			14
Race = Hispanic			2
Sex = Male			16
Primary Substance = Cocaine only			3
Primary Substance = Crack cocaine			4
Primary Substance = Cocaine +			9

In **Table-2** we have also included genotype data from a fMRI study in China evaluating the effects of Synaptose™ in ten heroin addicted Chinese males. **Table-2** provides demographic information pertaining to this group. Diagnosis of heroin dependence was also determined in this group using DSM-IV criteria and other behavioral instruments. There were 10 Males and 0 females with a median age of  $33 \pm 7.6$  SD years. The population breakdown was as follows: 100 % Chinese. The average

number of months abstinent for the entire population was  $16 \pm 7.9$ . There were 10 pure heroin only addicts.

## 2.2 Genotyping

A brief description of the genotyping methods for the polymorphisms to be assayed in this project follows. All methods are routinely performed in the Institute of Behavioral genetics (IBG), Boulder, Colorado laboratory. Each patient was also genotyped for the following gene polymorphisms: **MAOA-VNTR**, **5HTTLPR**, **SLC6A3**, **DRD4**, **ANKKI**, **DRD2 TaqIA (rs1800497)** and the **COMT val<sup>158</sup>met SNP (rs4680)**. Genotypes were scored by two investigators independently.

**The dopamine transporter (DAT1, locus symbol SLC6A3**, which maps to 5p15.3, contains a 40 base-pair Variable Number Tandem Repeat (VNTR) element consisting of 3-11 copies in the 3' untranslated region (UTR) of the gene [66]. The assay [67] is a modification of the method of Vandenberg *et al.* [66]. Primer sequences were: Forward- 5'-TGTGGTGTAGGGAACGGCCTGAG-3'; and Reverse- 5'-CTTCCTGGAGGTCACGCT CAAGG-3'.

**Table 2. Demographics of all Chinese subjects combined\***

	Median ±st.dev.	(min, max)	N (total = 10)
Age	33 ± 7.57	(20, 44)	10
Clean time	16 ± 7.91	(1, 24)	10
Race =			10
Sex = Male			10
Primary Substance = Heroin only			10
Primary Substance = Heroin +			0

\*One sample was eliminated because of low amplification so that genotyping was not possible.

**The dopamine D4 receptor (DRD4)**, which maps to 11p15.5, contains a 48 bp VNTR polymorphism in the third exon [68], which consists of 2-11 repeats. The assay [67] is a modification of the method of Lerman, *et al.* (1998) [69]. Primer sequences were: Forward- 5'-VIC -GCT CAT GCT GCT GCT CTA CTG GGC-3'; and Reverse-5'-CTG CGG GTC TGC GGT GGA GTC TGG-3'.

**Monoamine Oxidase A upstream VNTR (MAOA-uVNTR)**: The MAOA gene, which maps to Xp11.3-11.4, contains a 30 bp VNTR in the 5' regulatory region of the gene which has been shown to affect expression [70]. A genotype by environment interaction has been reported for this polymorphism [71]. The MAOA-u VNTR assay is a modification [72] of a published method [70]. Primer sequences were: Forward- 5'-ACAGCCTGACCG-TGGAGAAG-3'; and Reverse- 5'-GAACGTGACGCTCCATTCCGGA-3'.

**Serotonin Transporter-Linked Polymorphic region (5HTTLPR)**: The serotonin transporter (**5HTT, Locus Symbol SLC6A4**), which maps to 17q11.1-17q12, contains a **43 bp insertion/deletion (ins/del)** polymorphism in the 5' regulatory region of the gene [73]. Due to an error in sequencing this was originally thought to be a 44 bp deletion. The long variant (**L**) has approximately three times the basal activity of the short promoter (**S**) with the deletion [74]. Primer sequences were: Forward- 5'-6FAM - ATG CCA GCA CCT AAC CCC TAA TGT - 3'; Reverse- 5'- GGA CCG CAA GGT GGG CGG GA - 3'.

Hu *et al.* (2005) [75] reported that a SNP (rs25531, A/G) in the Long form of 5HTTLPR may have functional significance: The more common L<sub>A</sub> allele is associated with the reported higher basal activity, whereas the

less common L<sub>G</sub> allele has transcriptional activity no greater than the S. The SNP rs25531 is assayed by incubating the full length PCR product with the restriction endonuclease MspI.

For all of the above VNTR and ins/del polymorphisms, PCR reactions contained approximately 20 ng of DNA, 10% DMSO, 1.8 mM MgCl<sub>2</sub>, 200 μM deoxynucleotides, with 7'-deaza-2'-deoxyGTP substituted for one half of the dGTP, 400 nM forward and reverse primers and 1 unit of AmpliTaq Gold® polymerase, in a total volume of 20 μl. Amplification was performed using touchdown PCR [76]. After amplification, an aliquot of PCR product was combined with loading buffer containing size standard (Genescan 1200 Liz) and analyzed with an ABI PRISM® 3130 Genetic Analyzer. Genotypes were scored by two investigators independently.

**DRD2 TaqIA (rs1800497)**: The gene encoding the dopamine D2 receptor maps to 11q23, and contains a polymorphic TaqI restriction endonuclease site located within exon of the adjacent ANKK1 gene which was originally thought to be located in the 3' untranslated region of the gene. The A1 allele has been reported to reduce the amount of receptor protein [77]. This SNP is done using a Taqman (5'Nuclease) assay [78]. Primer and probe sequences were: Forward primer- 5'-GTGCAGCTCACTCCATCCT-3'; Reverse primer- 5'-GCAACACAGCCATCCTCAAAG-3'; A1 Probe- 5'-VIC-CCTGCCTTGACCAGC-NFQMBG-3'; A2 Probe- 5'-FAM-CTGCCTCGACCAGC-NFQMBG-3'.

**COMT val<sup>158</sup>met SNP (rs4680)**: The gene encoding Catechol-O-methyltransferase (COMT) maps to 22q11.21, and codes for both the membrane-bound and soluble forms [79] of the enzyme that metabolizes dopamine to 3-methoxy-4-hydroxyphenylethylamine [80]. An A→G mutation results in a valine to methionine substitution at codons 158/108, respectively. This amino acid substitution has been associated with a four-fold reduction in enzymatic activity [80]. The COMT SNP is assayed with a Taqman [78] method. Primer and probe sequences were: Forward Primer- 5'-TCGAGATCAACCCCGACTGT-3'; Reverse Primer- 5'-AACGGG-TCAGGCATGCA-3'; Val Probe- 5'-FAM-CCTTGCTCCTTCACGCCAGCGA- NFQMBG-3'; Met Probe- 5'-VIC-ACCTTGCTCCTTCATGCCAGCGAAAT- NFQMBG-3'.

Details, including primer sequences and specific PCR conditions may be found in Anchordoquy *et al.* [67], Haberstick and Smolen [78] and Haberstick *et al.* [72].

## 2.3 Addiction Risk Score

In terms of genotyping data we have determined based on literature review that there are seven risk alleles involved in the six candidate genes studied in this patient population. To determine severity of the 25 patients studied (one Chinese subject was eliminated from the analysis due to poor PCR amplification) we calculated the percentage of prevalence of the risk alleles and provided an arbitrary severity score based on percentage of risk alleles present. Subjects that carry the following alleles: DRD2=A1; SLC6A3 (DAT) =10R; DRD4=3R or 7R; 5HTTIRP = L or L<sub>A</sub>; MAO = 3R; and COMT=G. As depicted in **Table- 2** Low Severity (LS) = 1-36%; Moderate Severity (MS) =37-50%, and High Severity (HS) = 51-100%.

## [III] RESULTS

The resultant genotyping is illustrated in **Table-3** of this report and represents a total of 16 patients (group1) identified as not only addicts but the type of drug of choice.



**Table: 3. Group 1 Resultant genotyping data for each Caucasian patient.**

Subject #	MAOA uVNTR	5HTTLPR	5HTTLPR	SLC6A3	DRD4	DRD2	COMT	Any risk allele	SEVERITY* ARS
1	3R	S/L	S/LG	9R/10R	4R/4R	A1/A2	G/G	POSITIVE	0.46-MS
2	3R	S/L	S/LA	10R/10R	4R/7R	A2/A2	G/G	POSITIVE	0.62 -HS
3	3R	L/L	LA /LG	9R/9R	3R/4R	A1/A2	A/G	POSITIVE	0.57-HS
4	4R	S/L	S/LA	10R/10R	3R/7R	A2/A2	G/G	POSITIVE	0.46-MS
5	4R	L/L	LA/LA	10R/10R	4R/7R	A2/A2	A/G	POSITIVE	0.62 -HS
6	3R	S/S	S/S	9R/10R	4R/7R	A2/A2	A/G	POSITIVE	0.30 -LS
7	4R	S/L	S/LG	10R/10R	4R/4R	A1/A1	A/A	POSITIVE	0.38 -MS
8	4R	S/L	S/LA	9R/10R	3R/4R	A2/A2	A/A	POSITIVE	0.23-LS
9	3R	L/L	LA/LA	9R/9R	4R/7R	A2/A2	A/G	POSITIVE	0.54-HS
10	4R	L/L	LA/LA	9R/10R	4R/4R	A2/A2	G/G	POSITIVE	0.54 -HS
11	3R	S/L	S/LA	9R/10R	4R/4R	A1/A2	G/G	POSITIVE	0.54-HS
12	4R	L/L	LA/LA	9R/10R	4R/4R	A1/A2	A/G	POSITIVE	0.54-HS
13	4R	S/L	S/LA	10R/10R	4R/4R	A1/A2	A/G	POSITIVE	0.46 -MS
14	4R	S/S	S/S	9R/10R	4R/4R	A1/A2	G/G	POSITIVE	0.30-LS
15	3R	L/L	LA / LA	10R/10R	4R/4R	A1/A2	A/G	POSITIVE	0.69 -HS
16	4R	S/L	S/LA	10R/10R	4R/7R	A1/A2	A/A	POSITIVE	0.46-MS

**Severity percentage: LS =19; MS=31; HS= 50**  
**Average GARS score: LS= 0.28; MS=0 .44; HS =0 .58**  
**Prevalence of DRD2 A1 allele = 56%**  
**Percentage of Moderate and High Severity= 81**

In terms of genotyping data we have determined based on literature review that there are seven risk alleles involved in the six candidate genes studies in this patient population. To determine severity of the 16 patients studied we calculated the percentage of prevalence of the risk alleles and provided an arbitrary severity score based on percentage of risk alleles present. Subjects that carry the following alleles: DRD2=A1; SLC6A3 (DAT)=10R; DRD4=3R or 7R; 5HTTIRP = L or LA; MAO= 3R; and COMT=G. As depicted in [Table-2](#) low severity (LS) = 1-36%; Moderate Severity MS) =37-50%, and High

Severity (HS) = 51-100%. Based on this model 16 subjects tested have at least one risk allele or 100%. Out of the 16 subjects we found 50 % (8) HS; 31% (5) MS; and 19% LS (3 subjects). These scores are then converted to a fraction and then represented as an GARS whereby we found the average GARS to be: 0.28 Low Severity; 0.44. moderate severity and 0.58 high severity respectively. Therefore, using GARS we found that 81 % of the patients were at moderate to high risk for addictive behavior. Of particular interest we found that 56 % of the subjects carried the DRD2 A1 allele (9/16) [[Table-3](#)].

**Table 4. Group 2 Resultant genotyping data for each Chinese patient.**

Subject #	MAOA uVNTR	5HTTLPR	5HTTLPR	SLC6A3	DRD4	DRD2	COMT	Any risk allele	SEVERITY* ARS
1	4R	S/L	S/LA	10R/10R	4R/4R	A2/A2	A/A	POSITIVE	0.30-LS
2	3R	S/S	S/S	10R/10R	2R/4R	A1/A2	GAG	POSITIVE	0.38-MS
3	4R	S/S	S/S	10R/10R	3R/4R	A1/A2	G/G	POSITIVE	0.46-MS
4	3R	S/S	S/S	10R/10R	4R/6R	A2/A2	G/G	POSITIVE	0.38-MS
5	4R	S/S	S/S	10R/10R	4R/4R	A1/A2	A/G	POSITIVE	0.30-LS
6	3R	L/L	S/LG	10R/10R	4R/4R	A1/A2	ND	POSITIVE	0.45 -MS
7	4R	L/L	LA/LG	10R/10R	4R/4R	A1/A2	A/G	POSITIVE	0.54 -HS
8	4R	S/S	S/S	10R/10R	4R/5R	A2/A2	A/G	POSITIVE	0.23-LS
9	3R	S/L	S/LA	10R/10R	2R/4R	A2/A2	A/G	POSITIVE	0.46-MS

**Severity percentage: LS =33; MS=56; HS= 11**  
**Average GARS score: LS= 0.28; MS=0 .43; HS =0 .54**  
**Prevalence of DRD2 A1 allele = 56%**  
**Percentage of Moderate and High Severity= 67**

Moreover, data obtained from an on-going fMRI study in China (YL and JT) in nine heroin addicted males [see demographic [Table-2](#)] show similar genotype data [[Table-4](#)]. Based on this

model 9 subjects tested (Group 2) have at least one risk allele or 100%. Out of the 9 subjects we found 11% (1) HS; 56% (5) MS; and 33% LS (3 subjects). These scores are then converted

to a fraction and then represented as an GARS whereby we found the average GARS to be: 0.28 Low Severity; 0.43 moderate severity and 0.054 high severity respectively. Therefore, using GARS we found that 67 % of the patients were at moderate to high risk for addictive behavior. Of particular interest we found that 56 % of the subjects carried the DRD2 A1 allele (5/9) [Table-4]. Statistical analysis revealed that the groups did not differ in terms of overall severity (67 vs. 81%) in these two distinct populations. Using the z-test of proportions, the resulting  $z=0.79$  with  $p=0.432$ . However a sample size of 228 for Group 1 and 128 for Group 2 to detect a significant difference between two populations with 81% and 67% risk by z-test at the 0.05 level with power of 80%.

Nevertheless, combining these two independent study populations (Group 1 and Group2) reveal that subjects entering a residential treatment facility for poly-drug abuse carry at least one risk allele (100%). We found that 74% of this combined 25 subjects (Caucasian and Chinese) had a moderate to high GARS.

#### [IV] DISCUSSION

While this exploratory study did not carry out any specific statistical analysis such as Bayesian Theorem, Structural Equation Modeling or Recursive Partitioning (PR) the subject of work in progress the study was still informative. In terms of genotyping data we have determined that when multiple candidate genes are analyzed such as serotonergic- 2A receptor (5-HTT2a); serotonergic transporter (5HTTLPR); (dopamine D2 receptor (DRD2), Dopamine D4 receptor (DRD4); Dopamine transporter (DAT1); Catechol-o-methyl –transferase (COMT), and monoamine –oxidase (MOA) genes we found that 100% of all subjects carried at least one risk allele. Moreover this is the first time that anyone attempted to stratify or classify addiction risk by incorporating an algorithm formulation of combining a number of risk alleles by pre-assigning an allele as an risk allele having predictive value for drug use. For example it has been published earlier that the DRD2 A1 allele had a predictive value for all Reward Deficiency Syndrome (RDS) behaviors using Bayesian statistics to have a high predictive value of 74.4%. [3] and reviewed by Bowirrat *et al.* [81]. It is of further interest that the subjects studied in this investigation had multiple drug abuse relapses and presented to in-patient residential treatment programs. Our preliminary finding of approximately 75% of these individuals having moderate to high GARS whereby only 25% had low GARS suggest a potential utility for pre-screening patients prior to a one-size fits all treatment plan. Clinically this may have real importance in understanding expectations of future success and the need for intensive treatment involving genomic solutions coupled with bio-holistic medical therapies [82].

The present exploratory study supported the hypothesis suggested earlier by us and others [60, 83] by identifying hypodopaminergic genotypes as the best predictor of drug abuse

behavior in an adult and even more so in an adolescent population. This work is in agreement with Melis *et al.* [11] that identified a hypodopaminergic state as a causal mechanism in the development of SUD. This is consistent with a number of functioning Magnetic Resonance Imaging (fMRI) studies showing the importance of DRD2 levels by genotyping indicating that hypodopaminergic A1 genotype leads to blunted response and as such could lead to aberrant drug and or food seeking behavior [84, 85] while hyperdopaminergic A2 genotype serves as a protective factor against the development of drug disorders [86].

A further strength of this study is that we only used male subjects. de Courten –Myers *et al.* [87] have pointed out that one of the difficulties in replicating single gene associations with drug use disorder is sex –based or gender differences in neuro –chemistry and neuroanatomy. Moreover, Conner .*et al.* [60] suggested that males with hypodopaminergic functioning are more likely to abuse drugs that stimulate the mesocortical limbic system than those with normal dopaminergic functioning. In contrast, females living in a negative environment are at increased risk (possibly not due to their genotypes) for using more drugs and even more types of drug which increase their risk for SUD.

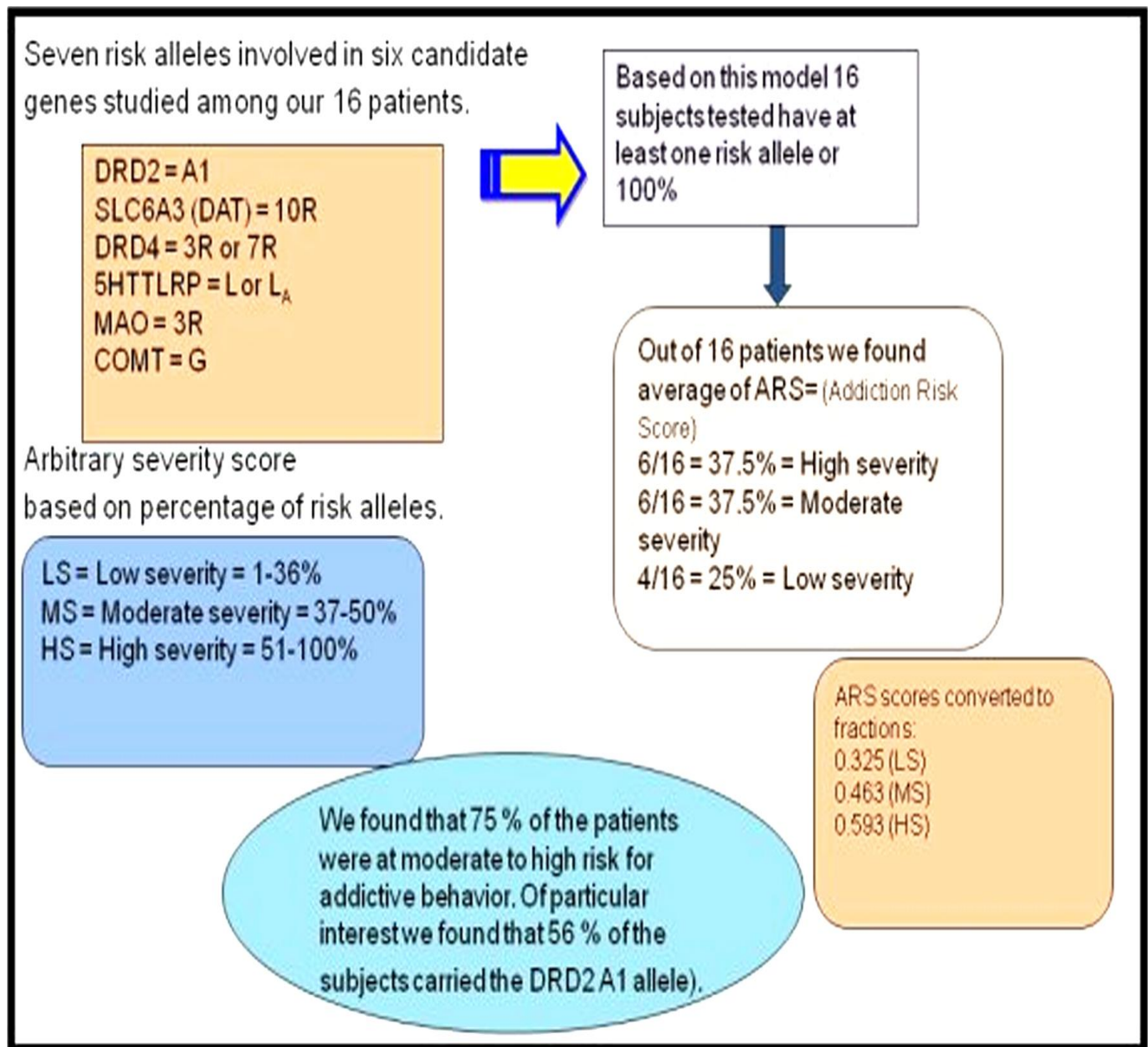
Another strength of this exploratory study is that it is in agreement with the work of Conner *et al.* [60] confirming the importance of the cumulative effect of multiple genotypes coding for hypodopaminergic functioning, regardless of their genomic location, as a predictive method of drug use in males. Moreover, it extends the current literature, by suggesting for the first time a simple method using genetic testing to classify risk behavior in male patients seeking in-patient residential treatment.

The limitations of this study must be considered before interpreting the findings. This was only an exploratory study and as such a small sample size was utilized to obtain very preliminary data. This study showing positive association of a number of hypodopaminergic gene polymorphisms with drug abusing adults requires replication in a much larger population in both in-patient and out-patient facilities. The confirmatory studies must include both males and females. The studies should extend the population base to specific drugs of choice, ethnic groups, age and other risk taking behaviors. Certainly the frequency of drug seeking behavior must also be considered in future experiments. Using a SUD scale [88] may also improve the generality of these findings. Most importantly many more candidate genes should be included in the GARS panel. Blum *et al.* [89] has reported on a so called “Happiness Gene Map which includes a total of 30 genes. These genes influence how reward is interpreted in the brain .Another impotent caveat is that the expression of these gene polymorphisms may be significantly impacted by epigenetic effects due to environmental elements.

While it is understood that future work will analyze the best predictive candidate genes to secure a predictive GARS panel

of genes utilizing a number of statistical tools such as recursive partitioning and Bayesian predictive modeling techniques the need for such a genetic test in the Chemical Dependence field seem parsimonious. A major limitation herein is that larger sample size and the definitive association of these risk alleles with validated severity scales (i.e. treatment response, failures and number of years addicted) are warranted. There are at least three practical reasons for such a diagnostic test: 1) identifying those at risk prior to the onset of SUD providing early intervention and prevention of the negative outcomes from such use; 2) removal of denial and guilt and 3) genotype results could suggest different at risk individuals and programs could be tailored to a patients risk profile.

It is important to note that the severity of risk in the Caucasian seemed to be somewhat different when we only look at the percentage of high GARS. Specifically, 50 % of the psychostimulant drug of choice dependent individuals (Caucasian) had a high GARS whereas only 11% of the Heroin addicted males (Chinese) had a high GARS. We do not have a reasonable explanation for this difference. However when both moderate and High GARS are combined for both groups we find that a total of 74% of these poly-drug abusers have a moderate to high GARS.



**Fig. 2. Genetic Addiction Risk Score (GARS) Analysis:** Exploratory Development of polymorphic risk alleles among 16 addicted patients. The figure does not display the results obtained for the Chinese samples.

## [V] CONCLUSION

The need to genetically test individuals especially at entry into a residential or even non-residential chemical dependency program has been suggested by scientists and clinicians alike here and abroad. In fact the most recent work of Conner *et al.* [60] has suggested the importance of multiple hypodopaminergic gene polymorphisms as a possible predictive tool to identify children at risk for problematic drug use prior to the onset of drug dependence. Our current exploratory study of only 16 Caucasians [as summarized in **Figure-2**] is in agreement with this prediction in terms of the development of a novel genetic test using an algorithm to determine the proposed GARS. To reiterate we found a high percentage (75%) of subjects carry a moderate to high GARS whereby 100% of individuals tested possess at least one risk allele tested. It is of some interest that in the Chinese population Group 2 only we found rare DRD4 alleles in this population such as 2R, 5R and 6R.

We are proposing, it is possible that the hypodopaminergic state is due to gene polymorphisms as well as environmental elements including both stress and neurotoxicity from aberrant abuse of psychoactive drugs (i.e. alcohol, heroin, cocaine etc). Genetic variables could include serotonergic genes (serotonergic receptors [5HT<sub>2a</sub>]; serotonin transporter 5HT<sub>1PR</sub>); endorphinergic genes (mu OPRM1 gene; proenkephalin (PENK) [PENK polymorphic 3' UTR dinucleotide (CA) repeats]; GABergic gene (GABRB3) and dopaminergic genes (ANKKI Taq A; DRD2 C957T, DRD4 7R, COMT Val/met substitution, MAO-A uVNTR, and SLC3 9 or 10R). Any of these genetic and or environmental impairments could result in reduced release of dopamine and or reduced number of dopaminergic receptors.

We are proposing that following needed confirmation positive outcome of GARS will have prevention and treatment benefits in those probands afflicted with genetic antecedents to RDS seeking behaviors.

## FINANCIAL DISCLOSURE

The following authors have financial conflicts based on patented technology related to the Genetic Addiction Risk Score (GARS) gene panel which has been licensed by Kenneth Blum to LifeGen, Inc. San Diego, California: Kenneth Blum, Roger L. Waite, B William Downs, Margaret Madigan, Abdalla Bowirrat, and David Miller.

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## RESOURCE LINKS

The following Web site links are suggested for additional information: <http://www.addictionsearch.com>; <http://www.drugstrategies.org> and <http://www.rdsyndrome.com>

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