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Risk factors for cannabis use disorders in Caucasian and American population

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Abstract

Marijuana is the most commonly used illicit drug globally and its use is associated with dependence, craving, and drug-seeking behaviour. Compared with other drugs of abuse, less is known about the susceptibility to cannabis use disorders. Hence, the aim of the present work was to identify potential genetic and environmental risk factors associated with cannabis use, comparing a group of marijuana users and control subjects in both Caucasian (93 controls and 92 marijuana users) and American (96 controls and 40 marijuana users) populations. Genetic association studies on polymorphisms involved in dopaminergic and endocannabinoid pathways were performed through gel electrophoresis based analysis and TaqMan genotyping Assay (Thermo). Psychometric tests (ACES, CECA-q, PBI) and a sociodemographic data analysis (gender, marital status, level of education, BMI evaluation) were used to assess environmental influences on cannabis use in the Caucasian and American groups respectively. MeDIP-qPCR finally investigated DNA methylation variations that can alter or trigger drugs of abuse response in the American population.

In Caucasian population differences in allelic frequencies and genotype distributions of the SNP rs1800497 (Taq1A) ($p<0.03$) of ANKK1 gene were observed and heterozygous G/A carriers were found more frequent among marijuana users than controls ($p<0.051$) for the SNP rs1049353 (G1359A) of CNR1 gene. In addition, G allele of the SNP rs1049353 may represent a risk factor for cannabis use in American population, since G allele ($p<0.002$) and homozygous GG genotype ($p<0.01$) were found significantly higher in marijuana users compared to control subjects.

In American population differences were also observed in the genotypes distribution ($p<0.058$) for the SNP rs2501431, CNR2 gene. In our study, homozygous A/A genotype was found more frequent in the marijuana group compared to controls.

Using logistic regression models the psychometric variables were evaluated in Caucasian population and the *optimal parenting* was observed as protective factor against cannabis use. *Emotional neglect* and *physical neglect* were confirmed as specific risk factors to this condition. In American population, an increase in education by one level drops the risk of marijuana use by approximately half. In both the populations, the gender difference affected cannabis use, with males tending to use at a higher rate than females.

DNA methylation status was found significantly higher in marijuana users compared to control subjects in two of the genes analyzed: hypermethylation at the exon 8 (+66.7 kb from TSS) of DRD2 gene ($p<0.034$) and hypermethylation at the CpG-rich region, +3 kb from TSS, in the NCAM1 gene ($p<0.0004$).

This study is one of the first to investigate the association between environmental factors, genetic polymorphisms, DNA methylation and marijuana use. Further to explore and replicate these results would be important to understand if genetic polymorphisms, stressful life events and differences in DNA methylation of marijuana-associated genes could be used as biomarkers for the prevention and treatment of marijuana use disorders.

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Abbreviations

AC	Adenylate cyclase
ACEs	Adverse Children Experience Scale
ADHD	Attention deficit and hyperactivity disorder
AEA	Anandamide
ANKK1	Ankyrin repeat and kinase domain containing 1 gene
BDNF	Brain-derived neurotrophic factor
BMI	Body Mass Index
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
CBD	Cannabidiol
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CECA-q	Child Experience of Care and Abuse Questionnaire
CNR1	Cannabinoid receptor 1 gene
CNR2	Cannabinoid receptor 2 gene
CNS	Central nervous system
CTRL	Control
COMT	Catechol-o-methyltransferase
CRE	Calcium/cAMP responsive element
CREB	cAMP response-element binding protein
DA	Dopamine
DAT	Dopamine transporter gene
DRD2	Dopamine receptor 2 gene
D2R	Dopamine receptor 2
DRD4	Dopamine receptor 4 gene
D4R	Dopamine receptor 4
DNMT	DNA methyltransferase
DSM	Diagnostic and Statistical Manual of Mental Disorders
eCB	Endocannabinoid
ERK	Extracellular signal–regulated kinase
FAAH	Fatty acid amide hydrolase
gDNA	Genomic DNA
GEE	Generalized Estimating Equations
GPCR	G protein–coupled receptor
GABA	γ-aminobutyric acid
GABRA	Gamma-amino-butyric acid receptors gene
GWAS	Genome wide association studies
HDAC	Deacetylases
HPA axis	Hypothalamic–pituitary–adrenal axis
MeCP2	Methyl-CpG binding protein 2
MeDIP	Methylated DNA immunoprecipitation
miRNA	MicroRNA
MJ	Marijuana
NAc	Nucleus accumbens
NCAM	Neural Cell Adhesion Molecule
ncRNAs	Non-coding RNA
NMDA	N-methyl-D-aspartate receptor
NO	Nitric oxide
PBI	Parental Bonding Instrument
PCR	Polymerase Chain Reaction
PFC	Prefrontal cortex
PKA	Protein kinase A

PTSD	Posttraumatic Stress Disorder
SNP	Single nucleotide polymorphism
TET	Ten-eleven translocation (TET) enzyme
THC	Delta-9-tetrahydrocannabinol
VNTR	Variable number tandem repeats
VTA	Ventral tegmental Area
UTR	Untranslated region
2-AG	2-arachidonylglycerol
5-mC	5-methyl-cytosine
5-hmC	5-hydroxymethylcytosine

1.Introduction

1.1 Marijuana

Marijuana is a substance derived from leaves and flowers of the plant *Cannabis sativa*. A compressed and more potent form of Cannabis sativa is hashish, a sticky resin obtained from the female plant flowers (Figure 1). Cannabis sativa plant contains many active constituents that can interact with one another and more of them have been discovered in the last few years.

Figure 1 Cannabis sativa; on the right, marijuana derived from dried leaves and hashish.

The compounds identified in the plant so far consist of about 750 chemicals, 104 of which are cannabinoids (Radwan et al., 2015). Cannabinoids include delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN). The main psychoactive (mind-altering) chemical is delta-9-tetrahydrocannabinol (THC), found in a resin on the flowers and leaves of the female plant, belonging to the class of Cannabis constituents C21 terpenophenolic cannabinoids (Elsohly and Slade 2005). Most of the other cannabinoids are either inactive or only weakly active, although some, such as CBD, may modify the psychoactive effects of THC (Abood & Martin, 1992; Mechoulam & Hanus L, 2012). Cannabinoids refer to all the chemical compounds that act on cannabinoid receptors modulating neurotransmitter release in the brain. These include phytocannabinoids, compounds produced by plants *Cannabis sativa* or *Cannabis indica*, endocannabinoids (eCBs), neurotransmitters produced in the brain or in peripheral tissues that act on cannabinoid receptors, as well as synthetic cannabinoids that are structural analogs of phytocannabinoids (WHO publication, 2016).

1.1.1 History of Marijuana use

Marijuana has a long history and has been used since ancient times for achieving euphoria. The first documents that describe its use come from China and India and were reported in a Chinese medical reference from the twenty-eighth century B.C. (Mikuriya, 1969). Some documents report possible knowledge of the plant even since the time of the Egyptians (Russo et al 2007). Around A.D. 500, its use reached North Africa and Europe and its analgesic and intoxicating properties were known. In particular, hashish, spread quickly throughout 12th century in Iran and North Africa. Later in 1500, marijuana spread to America. The therapeutic use of cannabis was reported around 1800 in India and the drug was used, in addition, for recreational and religious purposes (Mikuriya, 1969). Its use has progressively increased over the last centuries (Walton, 1983). At the beginning of the 21th century, after more than a decade of

decreasing use, because of its classification as a substance of high abuse potential drug and of no accepted medical use (Blatman, 1970), marijuana smoking is again on the rise.

1.1.2 Methods of marijuana administration

Marijuana can be smoked in hand-rolled cigarettes or pipes, water pipes, or cigars. Recently, marijuana has also been mixed with foods and marijuana-infused products, especially since it is used in some countries with medical purpose (NIDA, Report 15-3859). A predominance of marijuana use as blunts, spliffs, and mulled cigarettes use has been reported (Schauer et al., 2016).

1.1.3 Demographic data

According to the World Drug Report 2016 from the United Nations Office on Drugs and Crime (UNODC), cannabis is still the most widely used illicit drug in the world and its consumption is stable, with 3.8 per cent of the global population having used cannabis in the last 15 years (Figure 2). However, the use of cannabis has been increasing in North America and Western and Central Europe. Cannabis use begins typically at the ages of 15–25 years old and about 10% of people who use cannabis become daily users and another 20−30% use it weekly (WHO, 2016). In "High School and Youth Trends" report by National Institute of Drug Abuse (NIDA, Report 15-3859) shows that the majority of high school seniors do not think occasional marijuana smoking is harmful and, in 2015, 21.3% of high school seniors used marijuana in the past 30 days.

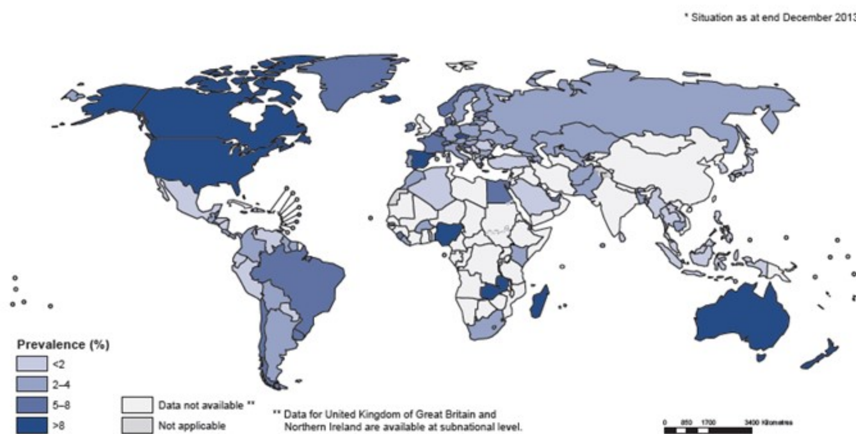


Figure 2. Annual prevalence of cannabis use for population aged 15−64 Years (World Drug Report 2015, UNODC).

European Drug Report 2016 shows that 1% of European adults are daily or almost daily cannabis users, 51.5 million males and 32.4 million females and the proportion of people seeking treatment for cannabis use disorders has been increasing in many regions of Europe. In addition, also the level of THC in both herbal cannabis and cannabis resin is increased and higher potency cannabis is now more widely available in Europe and the United States. The potency of cannabis depends on the growing conditions, the genetic characteristics of the plant, the ratio of THC to other cannabinoids, and the part of the plant that is used (Clarke & Watson, 2002). States that legalized medical marijuana in United States show higher rates of

marijuana use, but more research is needed to establish if the relationship is causal, because it is possible that there might exist common community norms that support both the legalization of medical marijuana and marijuana use (Cerdá et al., 2011).

1.2 Physical and pharmacological properties of marijuana

When marijuana is consumed in foods or beverages the THC amount in bloodstream is significantly less than used by other routes and its effects are also delayed, usually appearing after 30 minutes to 1 hour. This is due to the fact that the drug must first pass through the digestive system and necessitates consumption of higher amount of THC for comparable psychoactive effects of other routes of administration (NIDA, Report 15-3859). When marijuana is smoked, its psychoactive component, THC, travels throughout the body and reaches the brain faster. THC can also be detected in plasma within seconds of inhalation, with a half-life of 2 hours. Following smoking of the equivalent of 10–15 mg over a period of 5–7 minutes, peak plasma levels of THC are around 100 μg/L. It is highly lipophilic and widely distributed in the body. Some metabolites can be detected in the urine for up to 2 weeks following smoking or ingestion (Moffat et al., 2004). THC is a partial agonist at the cannabinoid receptor 1 (CB1) (Figure 3), which is widely expressed throughout the brain, with particularly high densities in the basal ganglia and substantia nigra (Herkenham et al., 1990).

THC and other cannabinoid chemicals in marijuana are similar to cannabinoid chemicals that naturally occur in the body. These endogenous molecules act as neurotransmitters on specific receptors and the network of the cannabinoid neurotransmitters, their cannabinoid receptors, and the enzymes involved in synthesis and degradation of endocannabinoids (eCBs) is called cannabinoid receptor system.

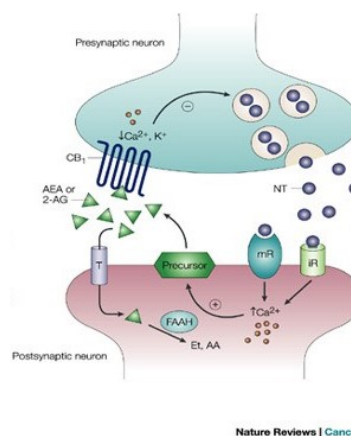


Figure 3. Endogenous cannabinoid system (Guzman, 2003).

1.2.1 The endocannabinoid system

Endocannabinoids are two small endogenous lipids (Figure 4), agonists for cannabinoid receptors: anandamide, an ethanolamine of arachidonic acid (AEA) (Devane et al. 1992), and 2-arachidonylglycerol (2-AG) (Mechoulam et al. 1995). Endocannabinoids are released from postsynaptic neurons by passive or facilitated diffusion; unlike the classical or peptide neurotransmitters, AEA and 2-AG are hydrophobic molecules synthetized if necessary as result of membrane depolarization due to calcium (Ca^{2+}) influx or by activation of $G_{q/11}$ protein-coupled receptors when high neuronal activity persists (Freund et al., 2003; Piomelli, 2003; Szabo

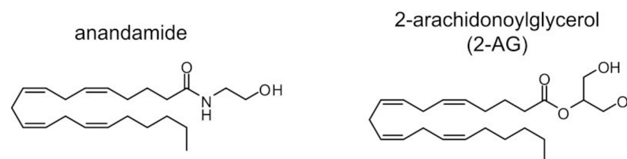


Figure 4. Chemical structures of endocannabinoids: Anandamide (AEA) (Devane et al. 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam et al. 1995).

& Schlicker, 2005). In more detail, postsynaptic neurons synthetize membrane-bound eCB precursors and cleave them to release active eCBs following an increase of cytosolic free Ca^{2+} concentrations, for example, after binding of neurotransmitters to their receptors (Piomelli, 2000) (Figure 6). The two cannabinoid receptors, CB1 and CB2 (Figure 5), are Gi/o protein–coupled receptor (GPCRs). The CB1 is the most abundantly expressed GPCR in the central nervous system (CNS) (Pertwee et al., 2010), and the highest transcription levels of CNR1 gene occur in the human brain, particularly in the regions involved in memory, emotional responses, cognition, motivation and motor coordination: hippocampus, amygdala, cortex, limbic forebrain and cerebellum respectively (Ceccarini et al., 2015; Hu and Mackie, 2015). Cannabinoid receptor 2 (CB2) expression is restricted mostly to microglia in the CNS, but is high in peripheral immune system (Galiegue et al., 1995; Munro et al., 1993).

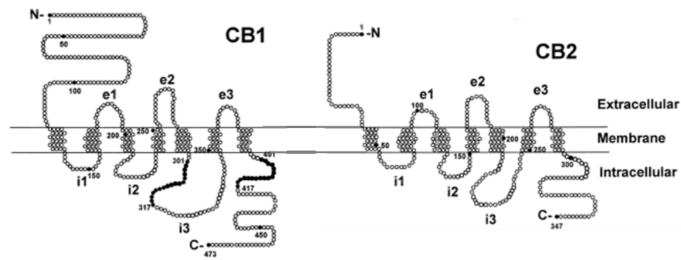


Figure 5. Cannabinoid receptors CB1 and CB2.

Endocannabinoids subsequently from the postsynaptic sites act as retrograde messengers by binding to presynaptic CB1 cannabinoid receptors. This communication from post to pre-synaptic neurons is called retrograde signaling (Wilson e Nicoll, 2002). They modulate protein kinase A (PKA) activity, through adenylyl cyclase inhibition with a consequentially decrease in the synthesis of second messenger cyclic adenosine monophosphate (cAMP). This event is coupled to the inhibition of voltage-sensitive Ca^{2+} channels and the activation of K^{+} channels, to maintain homeostasis and prevent the excessive neuronal activity (Wilson & Nicoll, 2001). Stimulation of CB1 receptor in vitro and in vivo activates extracellular signal–regulated kinase (ERK1/2) (Howlett, 2005).

The activation of CB1 receptors modulates the release of neurotransmitters such as glutamate, dopamine and γ-aminobutyric acid (GABA). These processes influence learning and memory, as well as movement (Schlicker & Kathmann, 2001). ECB-based neuromodulation concludes when these substances are rapidly removed from the extracellular space by cannabinoid transporter (Beltramo et al., 1997) in a reuptake mechanism, which ends their signalling by internalizing the molecule and allowing access to a family of intracellular degradative enzymes, the best characterized of which is fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996).

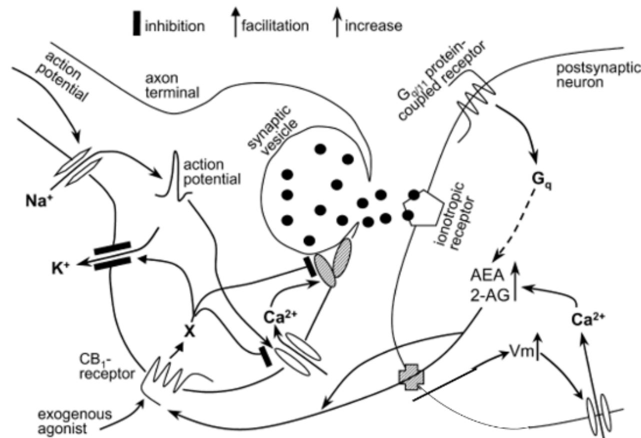


Figure 6. The endogenous cannabinoid system: after the activation of CB1 receptor (CB1), the presynaptic neurotransmitter release occurs in the inhibition of voltage-dependent calcium channels, activation of potassium channels and interference with vesicles release. CB1 can be activated from exogenous or endogenous agonists (Szabo & Schlicker, 2005).

The feedback inhibition mediated by eCBs is called "endocannabinoid-mediated plasticity" (Mackie, 2008) and it can either attenuate or enhance neuronal excitability, depending on the types of synapses: in the GABAergic synapse, the feedback mechanism will result in a depolarization-induced suppression of inhibition (DSI) whereas, in a

glutamatergic synapse, depolarization-induced suppression of excitation (DSE) occurs (Ohno-Shosaku & Kano, 2014; Volkow et a l., 2016).
The expression and magnitude of retrograde signaling, in addition, also show plastic capacity (Figure 7). That is because neuromodulators, neural activity, and co-released dendritic signals, confer plasticity to retrograde signaling and to the underlying signaling cascades (Iremonger et al., 2013).
In hippocampal pyramidal neurons enhanced activation of phospholipase C beta (PLCβ) and downstream eCBs synthesis increase GPCRs-mediated eCBs release (Gq GPCR), combined with postsynaptic calcium increases (Hashimotodani et al., 2005). Another mechanism of retrograde plasticity has been observed in striatal medium spiny neurons, where the activation of $G_{i/o}$ coupled receptors, such as dopamine D2 receptors (D2R), inhibits PKA and reduces the activation of regulator of G protein-signaling 4 (RGS4). This eCBs-dependent synaptic depression induces a greater activation of G_q GCPR pathways driving eCBs synthesis (Lerner & Kreitzer, 2012). In addition, the ability of central neurons to release dendritic transmitters has been demonstrated. For example, oxytocin magnocellular neurons constitutively release oxytocin, a hormone that plays a role in social bonding, sexual reproduction, and during/after childbirth (Yang et al., 2013).
Oxytocin drives the release of eCBs with autocrine and/or paracrine way (Hirasawa et al, 2004). Another retrograde signal is mediated by nitric oxide (NO).

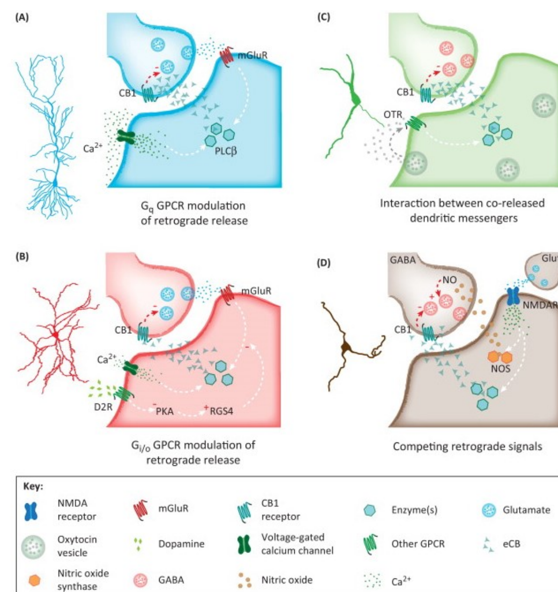


Figure 7. Four mechanisms of retrograde plasticity modulation and the underlying signaling cascades (Iremonger et al., 2013).

Glutamate release and the subsequent activation of NMDA receptors induce calcium influx in afferent axons in the dorsal medial hypothalamus. Calcium drives the release of eCBs and NO to decrease and increase GABA transmission, respectively (Crosby et al., 2011; Iremonger et al., 2013).
The functionality of CB1 receptors is also complex because of interactions with other neurotransmitter systems. For example, CB1 subunits can form heteromers with two or more GPCRs, especially when they are densely expressed in the same neuron, as the possibility of CB1 to form heteromers with D2R and/or adenosine has been demonstrated (Navarro et al., 2008).
Furthermore, evidence suggests that eCBs can affect neurodevelopment, specifically, the development of neuronal progenitor cell fate and of GABA interneurons; they can act as developmental signals indispensable for cortical neuron specification and help to create long distance connection (Berghius et al., 2007; Mulder et al. 2008). Through CB2 stimulation, eCB system plays a role in both the peripheral nervous system and in extraneural site, affecting gastrointestinal tract, liver, hurt, muscle, skin and reproductive organs (Maccarone et al., 2015) and controlling processes such as peripheral pain, vascular tone, intraocular pressure and immune function (Guzman, 2003). CB2 receptors can indeed modulate immune cell migration and cytokine release, small proteins responsible for many functions, including immune responses and inflammation, both outside and within the brain (Howlett, 2002; Saito et al., 2012). They are localized

predominantly in leukocytes, spleen, bone marrow and pancreas, but is expressed also by microglia and in subset of neurons, with increasing levels following injury (Atwood & Mackie, 2010).
ECBs can indirect affect dopamine signals. How much and how often dopamine is released from mesocorticolimbic dopamine neurons depends not only on dopamine transporter neurons, but also on the inhibitory neurons near the dopamine presynaptic cells (French et al. 1997), in neural regions such as the ventral tegmental area (VTA) and prefrontal cortex (PFC) (Laviolette 2007).

1.2.2 Delta-9-tetrahydrocannabinol: the cross-talk between endocannabinoid and dopaminergic systems

When someone smokes marijuana, THC molecule (Figure 8), because of its similarity to the eCBs AEA and 2-AG, competing with the brain eCBs, can bind cannabinoid receptors and activate them, affecting various mental and physical functions.

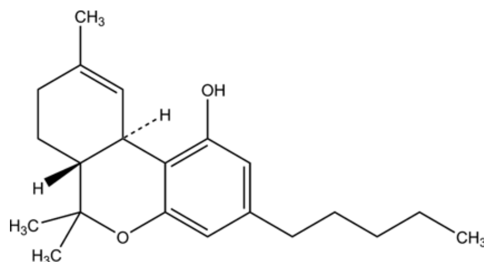


Figure 8. Delta-9-tetrahydrocannabinol (THC) (Mechoulam, 1970).

Cannabinoid receptors are responsible of different THC effects according to the synapse type: in glutamatergic synapses, they are responsible of THC effects in locomotion, hypothermia, analgesia, catalepsy, escaping behaviour; in the GABAergic synapses, they have a crucial role in deficit memory caused by THC, in stress and sensitivity to natural reward (Rossi et al., 2008; De Chiara et al., 2010).
Previous findings have shown evidence of cross-talk between the dopamine and eCB systems, indicating that these receptors respond to THC by increase dopamine release. THC produce a smaller dopamine release than cocaine or methamphetamines, but since THC is smoked, dopamine is more quickly released and this effect explains the euphoric effect of cannabis (WHO, publication 2016).
THC stimulates neuronal firing in the VTA and the nucleus accumbens (NAc) and increases striatal dopamine levels of mesolimbic dopamine neurons in animals (Lupica et al., 2004). In addition, it induces dopamine release in the human striatum (Bossong et al., 2009; Voruganti et al., 2001; Bossong et al., 2015; Sami et al., 2015), particularly severe cannabis dependence in human has been associated with deficit in the striatal dopamine release (Pistis et al., 2004; van de Giessen et al., 2016), but not all the studies confirm these results (Stokes et al., 2009; Barkus et al., 2011). Chronic cannabis use shows a decreased brain capacity to synthesize or release dopamine, a dose-dependent reduction in dopamine synthesis capacity in the corpus striatum (Bloomfield et al., 2014).

The effect of cannabis exposure on dopamine pathway and the reduced dopamine reactivity in the brain's reward regions can explain the hypothesis of cannabis gateway drug, which is the increased susceptibility to drug abuse and addiction to several drugs later in life (Volkow et al., 2014).

1.3 Effects of marijuana use

1.3.1 Is marijuana addictive?

About 10% of first-time cannabis users become addicted to the drug (MacDonald and Pappas, 2016) and subsequent health risks are often under-evaluated because of the misconception that marijuana is not addictive and is less harmless compared to other substances such as cocaine and heroin. Addiction is a condition where a person cannot stop using the drug even though he/she faces up with many negative consequences in his or her life. Perceptions of the risks of marijuana use, among teens, have declined over the past decade, possibly because of the debate about medicalization and legalization of recreational use (NIDA, Report 15-3859). Marijuana dependence occurs when the brain adapts to large amounts of the drug by reducing production of and sensitivity to its own eCB neurotransmitters (MacDonald and Pappas, 2016). A functional magnetic resonance imaging (fMRI) study shows the reward neurocircuitry activation associated with the neuropathology of addiction during craving for marijuana, in proportion with severity of cannabis-related problems (Filbey et al., 2009).

MacDonald and Pappas have provided a complete assessment of the three possible reasons that generated the misconception that cannabis addiction does not exist. First, the percentage of first-time use for other commonly abused drugs as stimulant (11%), alcohol (15%), cocaine (17%), heroin (23%), and nicotine (32%) is higher, compared to cannabis first-time use (9%) (van de Giessen et al., 2016). Second, THC half-life (25–57 hours) is long, suggesting that there might be less craving for THC (Grotenhermen F, 2003). Third, marijuana does not cause dramatic physical symptoms of withdrawal as heroin or cocaine, but more subtle symptoms such as anorexia, irritability, anxiety, anger, restlessness, and sleep disruption (Bachman et al., 1998; Bolla et al., 2008). One-third of regular users in the general population report cannabis withdrawal (Chung et al., 2008), demonstrated by the decrease of the withdrawal itself with cannabis or other substances use and the difficulty in quitting (Copersino et al., 2006; Hasin et al., 2013)

1.3.2 Short and long term effects of marijuana usage

Common short term effects of marijuana smoking are a pleasant euphoria and sense of relaxation, but depending on different users it also brings to heightened sensory perception, laughter, altered perception of time, and increased appetite. Some users experience anxiety, fear, distrust, or panic and large doses of marijuana can also cause an acute psychosis, which includes hallucinations, delusions, and a loss of the sense of personal identity.

Daily and near-daily cannabis use can result in more severe consequences. Long-term cannabis use effects include various issues related to cognitive function, social and educational outcomes, the use of other illicit drugs and in more severe cases mental illness (WHO, publication 2016).

Since the important role of eCB system in many brain and peripheral functions, the use and abuse of substances, as marijuana, that disrupt this endogenous system can affect dramatically nervous system and its development.

1.3.2.1 Effects of marijuana on early development

Despite it is difficult to study the effects of cannabis on child development, because confounding variables as the polydrug use, nutrition, and psychology factors, evidences suggest that prenatal cannabis exposure may interfere with normal development and maturation of the brain (WHO, publication 2016).
In the developing fetus the endogenous cannabinoid system plays an important role in cell migration and differentiation, neuronal migration, development of axonal pathways, and the creation of functional synapses and use of marijuana during pregnancy has been shown to disrupt the eCB system (Gaffuri et al., 2012; Sonon et al., 2015). Some studies have been shown the consequences of prenatal cannabis exposure to be fetal growth restriction (El Marroun et al., 2009), learning disabilities, memory impairment (Noland et al., 2005; Fried et al., 2005) increased hyperactivity, impulsivity, delinquency and inattention symptoms in the offspring (Goldschmidt et al., 2000) and higher likelihood of using cannabis later in adolescence and adulthood (Sonon et al., 2015).
In addition, the negative effects from marijuana use, as neuropsychiatric-like phenotypes and dependence, are confirmed to be related to the age of first exposure in both animal and human studies. In rats, THC injection prior to the onset of puberty makes the animals more susceptible to changes in emotional behaviour and stress situations (Silvia et al., 2016) and establishes other behavioral abnormalities.
In human, first exposure to cannabis could be a signal event leading subsequently to cannabis use disorder diagnosis in youths having high transmissible risk (an index that captures the intergenerational risk that is common to all substance use disorder, as well as anxiety, and numerous indicators of behavioural self-regulation) (Kirisci et al., 2013).

1.3.2.2 Effects during adolescence

Compared to people who start marijuana use in adulthood, adolescents are 2 to 4 times more likely to develop dependence within two years of their first use (Chen et al., 2009).
Early cannabis use appears to be associated with an anti-conventional lifestyle characterized delinquent and substance using peers, early school leaving, leaving the parental home and early parenthood (Lynskey and Hall, 2000).
The teenage and early adult years of human growth are critical stages for brain development, where stress, reward, and executive/regulatory circuits continue to develop (Koob & Volkov, 2009). During adolescence, the organization of the neuronal circuitry goes through a refinement: focusing on the mesocorticolimbic dopamine system, the PFC is still in development until early adulthood (Sowell et al, 1999; Gogtay et al, 2004; Shaw et al, 2008). It has been demonstrated in rodents that the dopamine innervations undergo a major reorganization to establish dopamine connectivity with modifications in fiber density and shape during adolescence (Kalsbeek et al, 1988; Reynolds et al., 2016).
And even local medial PFC neurons reach a mature state of responsiveness to dopamine during this period (Spear, 2000; Tseng and O'Donnell, 2007). Marijuana use is widespread particularly among adolescents and young adults and drugs of abuse, as cannabis, during adolescence, a crucial step in neuromaturation, may produce serious consequences, damaging the correct path of dopamine innervation to the medial PFC (Reynolds et al., 2016).
Given that, adolescents may be more vulnerable to potential consequences of marijuana use than adults (Schweinsburg et al., 2008). Scientific supports to this hypothesis come from different studies: focusing on an interval of up to 24 months following first drug use, the risk of addiction related problems is higher for adolescent recent-onset users of various drugs of addiction compared to adult recent-onset users (Chen et

al., 2008). These results are also confirmed on a specific study on alcohol, marijuana and cigarette use: early adolescent use of all the three substances is strongly associated with adult substance use problems (Moss et al., 2014).

From behavioral studies, repeated exposure to cannabis during adolescence seem to have a neurotoxic effect with a not full complete neuropsychological functioning restoration after cessation of use (Meier et al., 2012). These exposures may affect brain functional connectivity, intelligence, and cognitive function: adverse effects on IQ and a decrease across time between caudal anterior cingulate cortex and two frontal regions known to mediate executive function (left dorsolateral prefrontal cortex and orbitofrontal cortex) has been demonstrated (Camchong et al., 2016).

In a recent study, THC exposure, specifically during adolescence, has been shown to induce a state of hyper-dopaminergic function in the mesocorticolimbic system and alterations in several prefrontal cortical molecular pathways, persisting into early adulthood. However, the precise related mechanism is currently unknown (Renard et al., 2016).

1.3.2.3 Heavy usage marijuana effects

Based on these evidences many studies suggest a correlation of heavy marijuana use and mental illness, as an increased of risk of anxiety and depression (Patton et al, 2002), psychoses (including those associated with schizophrenia), especially among people with a pre-existing genetic vulnerability (Caspi et al., 2005). But it is challenging to understand how marijuana exacerbates clinical symptoms like psychosis and depression and which is the initial impact of marijuana use on schizophrenia (Wilson & Cadet, 2009). It is difficult to establish a causal role, since both mental illness and marijuana addiction are multifactorial condition (Volkow et al., 2015).

Cannabis exposure can also bring harmful effects in peripheral tissues. Acute exposure increases heart rate and blood pressure (Pacher & Kunos, 2013) with an increased risk of myocardial infarction and stroke (Thomas et al., 2014). Marijuana abuse was shown to be a risk factor for cardiovascular and cerebrovascular disorders (Aryana & Williams, 2007): the apolipoprotein, (apo) C-III, was found significantly increased in the serum of marijuana abusers (Jayanthi et al., 2010) and its upregulation might be a significant player in MJ-mediated vascular and neuroimaging abnormalities (Bolla et al., 2002; Lee et al., 2003).

Cannabis smoke seems to be also associated with inflammation of the large airways and increased symptoms of chronic bronchitis (Gates et al., 2014). Studies investigated also the risk of long-term marijuana smoking on lung cancer; however, confounders such as cigarette smoking made unclear the result (Hashibe et al., 2006).

Findings from neuroimaging studies of the effects of marijuana use on brain structures reported structural alterations in the brain of heavy marijuana users, smaller volumes in regions rich in CB1 receptors, which are the neocortex, thalamic nuclei, limbic regions, basal ganglia and cerebellar cortex (Glass et al., 1997). One of the most consistently findings is smaller hippocampus, amygdala volumes (Lorenzetti et al., 2015), changed activity within the striatum (Yip et al., 2014), denser grey matter in the parahippocampal gyrus and denser white matter in the left parietal lobe among heavy users compared to non-users (Matochik et al., 2005). One study showed also a correlation between genotypes and reduced hippocampal and amygdala volume, indicating a polymorphism of CNR1 gene as a predictor of lower volume of bilateral hippocampi among cannabis users relative to controls (Schacht et al., 2012).

Neuroimaging study, investigating the long-term neurocognitive deficits, have also found a reduced volume in cerebellum and frontal cortex (Batalla et al., 2013).

1.4 Marijuana use disorder

Despite the central role of consumption, until a few years ago, no specific or maladaptive pattern of cannabis use were defined and appeared as diagnostic criteria (Compton et al., 2009). In the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) cannabis abuse and dependence were defined as maladaptive patterns of cannabis use leading to clinically significant impairment or distress, as manifested by at least one of four abuse symptoms criteria or three of six dependence symptom criteria, respectively (American Psychiatric Association, 1994; Compton et al., 2009). Evaluating the validity and utility of diagnostic criteria of cannabis use disorders, the DSM-IV criteria reflected a range of cannabis problems from moderate to severe but did not consider mild manifestations of the disorder. In order to improve the spectrum severity of the substance use, Piontek, analysing an adolescent population sample, highlighted the need to add additional cannabis use indicators, in particular, daily use. Daily use, that can be evaluated as daytime use and using cannabis when being alone, was associated strongly with cannabis dependence (Piontek et al., 2011). Cannabis withdrawal was not included in DSM-IV because of a lack of evidence, but recently, changes to diagnostic criteria for substance use disorder in general and for the diagnosis of cannabis use disorders have been made in DSM-V (American Psychiatric Association, 2013), adding cannabis withdrawal as a criterion for cannabis use disorder, defined as craving or a strong desire or urge to use the substance (Hasin et al., 2013). Thus, according to DSM-V, cannabis use disorder, that begins most in early adolescence or as young adults and includes cannabis abuse and dependence, can be mild or moderate or severe, depending on the number of symptoms (American Psychiatric Association, 2013). Diagnostic Criteria provided by DSM-V are listed below:

A. Cessation of cannabis use that has been heavy and prolonged (i.e., usually daily or almost daily use over a period of at least a few months).
B. Three (or more) of the following signs and symptoms develop within approximately 1 week after Criterion A:
   1. Irritability, anger, or aggression.
   2. Nervousness or anxiety.
   3. Sleep difficulty (e.g., insomnia, disturbing dreams).
   4. Decreased appetite or weight loss.
   5. Restlessness.
   6. Depressed mood.
   7. At least one of the following physical symptoms causing significant discomfort: abdominal pain, shakiness/tremors, sweating, fever, chills, or headache.
C. The signs or symptoms in Criterion B cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
D. The signs or symptoms are not attributable to another medical condition and are not better explained by another mental disorder, including intoxication or withdrawal from another substance.

1.5 Marijuana use disorders as a multifactorial condition

Co-occurrence of genetic, environmental, and epigenetic factors may increase the susceptibility to marijuana use disorder.

1.5.1 Genetic studies

Identifying genetic factors that may interact with marijuana abuse is crucial to differentiate factors which contribute to susceptibility to this disorder from the toxic and adverse health effects of THC following marijuana use (Brumback et al., 2016).

1.5.1.1 Family, adoption and twin studies

The total variance in genetic risk of cannabis use disorders has been estimated in the region of between 30 and 80% (Agrawal & Lynskey, 2009). To evaluate how much substance use disorders may be influenced by heritable factors family, adoption and twin studies can be conducted (Hall et al., 2013). These studies highlight potential heritable component to marijuana use and abuse/dependence (Agrawal et al, 2007).
Adoption studies, not cannabis specific, but into drug and alcohol use, have found that abuse or dependence of adoptees is more related to abuse or dependence of their biological parents than their adoptive parents (Cadoret et al., 1995), indicating an important role for genetic factors.
Twin studies show that marijuana use before age 17 increased risk of abuse or dependence to other substances, as opioids sedatives cocaine or other stimulants, hallucinogens (Lynskey et al., 2003). Furthermore, the influence of common genetic factors on problematic cannabis use has been estimated to be 51% among males, 20% to a shared environment and 29% to an unshared environment. Among females, 59% was attributed to genetics, 15% to a shared environment, and 26% to an unshared environment (Verweij et al., 2010; WHO, publication 2016). Parental substance use negatively impact with physical, psychological and cognitive consequences for children's development (Bröning et al., 2012).
The limitations of these approaches are the impossibility to establish which genes are involved and the identification of the risk alleles, how these genes determine those effects and how genetic diversity influences the sensitivity to either adverse or protective environment.

1.5.1.2 Candidate gene association studies

The study of cannabis dependence should not focus on the impact of a single gene, but it should examine many genes networks and environmental conditions that work in concert affecting human behaviors (Hamer, 2002). The association can result from co-occurrence of risk nucleotide variations and not everyone has the same risk to develop addiction: population groups, because of genetic differences among individuals, can show a higher risk condition, commonly known as vulnerability. Since neurotransmitters and their receptors play a key role in drug addiction, susceptibility to drug dependence can be affected by genetic variants in genes involved in drugs of abuse metabolism or dopaminergic and eCB system. It has been estimated that protein-coding regions make up only approximately 1% of the human genome, the remaining 99% of the genome is important for regulating gene expression, RNA genes, transposons, heterochromatin and other sequences (ENCODE). Of all these genomic sequences humans share 99.9%; this small variation between individuals, including about 10 million of polymorphisms, determines the individual differences between us (Haddley et al., 2008). Polymorphisms are genetic sequence variations

present in the population with a frequency greater than or equal to 1%. They can be located in untranslated regions, affecting promoter activity, or in DNA coding sequences where they can result in missense substitution (resulting in a codon that codes for a different amino acid) or synonymous/silent substitution (the produced amino acid sequence is not modified, because of genetic code degeneration). Also synonymous mutations can affect transcription, splicing, mRNA conformation, and translation (Chamary et al., 2006). Polymorphic sequences can be single nucleotide polymorphisms (SNPs), when the difference between 2 alleles is due to one nucleotide in the DNA sequence exchanged for another (Figure 9). They occur about 1000 bp on the human genome. Other SNPs include omissions, where a nucleotide is absent in one sequence but not in another, or insertions, where an extra nucleotide is found in a DNA sequence. Focusing on predisposition to specific conditions, because of inheritance, many SNPs can occur together and SNPs which are always inherited linked together are known as haplotypes.

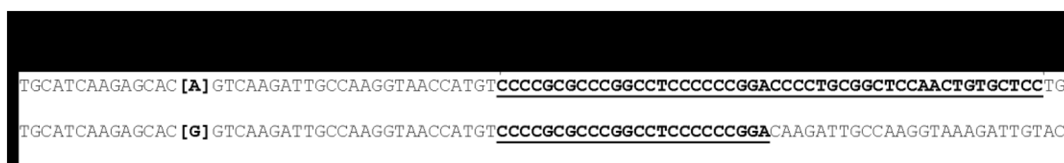


Figure 9. Main genetic polymorphisms:
single nucleotide polymorphisms (SNPs) and variable number tandem repeats (VNTRs)

The second type of polymorphism include variable number tandem repeats (VNTRs), when a sequence greater than 6 base pairs (bp) is repeated different times between alleles. The majority are located in non-coding regions and they seem to constantly evolve by changes in copy number. Since they present potential *cis*-regulatory elements or transcription factor binding sites they could modulate transcription or alter post transcriptional properties, as mRNA stability (Haddley et al., 2008). From the Human Genome Project to date a high-density map of genomic variations has been built and many information on SNPs and other polymorphisms are available for many genes, most involved in drug metabolism, immunity and inflammation (Bühler et al., 2015). Several of these are attractive candidates for disease susceptibility genes.

To identify specific genes/alleles involved in addiction, three strategies can be adopted: candidate gene association studies, modelling candidate genes with knockout mice and genome wide association studies (GWAS). Multifactorial and complex traits are influenced by many variants, each with very small effect sizes; in the same way cannabis use disorder is a highly polygenic trait, comprising many SNPs each with small effects contributing to susceptibility. Candidate gene approaches tend to focus upon specific sets of genes based upon a priori assumptions about the importance of the genes in addiction. Today the availability of detailed information on human mutations improve this study design and they have become important to the area of clinical pharmacology to develop of new drug treatments (Daly et al., 2001).

SNPs studies

The most probable candidate genes for cannabis use disorder are those encoding the two cannabinoid receptors, CNR1 and CNR2. Bühler and co-workers report, as the most significantly associated SNPs with cannabis related phenotypes, rs806380 of CNR1 and rs324420 of fatty acid amide hydrolase gene (FAAH), responsible mainly for degradation of the endogenous cannabinoid anandamide (Bühler et al., 2015). The

G allele of rs806380 SNP, located in intron 2 of CNR1, gives a protective effect in developing one or more cannabis dependence symptoms (Hopfer et al., 2006; Agrawal et al., 2009). Other SNPs of CNR1 have been studied related to substance dependence, for example, rs6454674 has been found significant associated to drug and alcohol dependence (Zuo et al., 2007) and in a haplotype analysis associated with cannabis related problems (Hopfer et al., 2007). Since none of these studies exclude cannabis dependence, defining drug dependence, we can still hypothesize that these variations might have a role in vulnerability to cannabis use disorders (Agrawal & Lynskey, 2009). The synonymous SNP rs1049353 (G1359A), resulting in the substitution of a guanine to adenine at nucleotide position 1359 in codon 435, has been reported in association with rs806380 to be a risk factor for the development of cannabis dependence (Hartman et al., 2009). An association between CNR1 and alcohol dependence is found in a haplotype analysis with SNPs rs6454674-rs1049353-rs806368 (Marcos et al., 2012).

A haplotype of three SNPs of CNR1, rs2023239-rs12720071-rs806368, has been associated with nicotine dependence (Chen et al., 2008). Significant rs12720071 genotype-by-marijuana use interaction effects on white matter volumes and neurocognitive impairment; in details, G-allele carriers compared to AA homozygotes, had smaller frontal and temporal white matter volumes that could be responsible of the phenotypic abnormalities in schizophrenia (Ho et al., 2011).

Another CNR1 SNP studied in CNR1 is rs806379; AA carriers present enhanced impulsivity in early adversity conditions, in association with the rs1049353 T allele (Buchmann et al., 2015). In addition, subjects with the T allele or T-positive genotypes show a rapid onset of psychosis after methamphetamine abuse (Okahisa et al., 2011)

G allele of rs2180619 SNP is found more frequent in subjects with substance use disorders (Zhang et al. 2004) and has been associated with high levels of anxiety; GG carriers, by interacting with the SS genotype of the promoter region of the serotonin transporter (5-HTTLPR) gene, were four-fold at risk for high anxiety (Lazary et al. 2009). Furthermore, GG subjects, compared to AA subjects show a general lower performance in the task difficulties in working memory tasks and (3) a higher vulnerability to distractors (Ruiz-Contreras et al., 2014).

Few studies have investigated variations in CNR2, encoding cannabinoid receptor 2, and addiction development. Rs2501431, a synonymous substitution in exon2, has been found associated with depression, with more severe type of depression for AA homozygous carriers than G carriers (Mitjans et al., 2012). In a study on genetic variation in the endocannabinoid system, children with a primary anxiety disorder diagnosis were recruited and the SNP rs2501431 resulted nominally associated with response in a subset with fear-based anxiety disorder diagnoses (fear-based anxiety disorder diagnosis included specific phobia, social phobia, separation anxiety disorder, panic disorder) (Lester et al., 2016). None of these researches investigate the possible correlation with cannabis use.

The second SNP reported from Bühler and co-workers was rs324420 of the FAAH gene, potentially significant because it is conserved from multiple mammalian species. This SNP has been found to impact on biochemical and cellular functioning of the enzyme, causing a change from the amino acid proline to threonine (P129T) (Chiang et al., 2004). Homozygous genotype A/A has been shown to confer higher propensity to toxicodependence (Sipe et al., 2002). However, focusing only on cannabis abuse in another study, A/A individuals were 0.25 times less likely to be cannabis-dependent; the authors hypothesized the reduced risk for cannabis dependence results from a diminished FAAH activity leading to increased levels of endogenous cannabinoids with consequent improvement in craving and withdrawal; the same subjects were potentially more likely to try cannabis and this effect could be explained because increased endogenous anandamide, as exogenous cannabinoids, reduces anxiety in mammals (Kathuria et al., 2003).

The missense variation P129T could increase risk of THC assumption, but not dependence (Tyndale et al., 2007). Recently, rs324420 was also found in moderate linkage disequilibrium with rs4141964, associated with cannabis use disorders in a young adult Mexican Americans population (Melroy-Greif et al., 2016).
Evidence of association between gene variations and marijuana use were found also in other genes. High-risk haplotypes of gamma-amino-butyric acid receptors gene (GABRA2) were found associated with cannabis dependence (Agrawal et al., 2006; Philibert et al. 2008). Other research report association of GABRA2 gene variations with alcohol dependence, but not with smoking, cannabis, or illicit drug use (Lind et al., 2008).
A wide variety of reports have been investigated genes coding proteins, transporters, receptors and enzymes involved in the dopaminergic mesocorticolimbic reward pathways eCB system. Since cannabis use has been demonstrated to increase the release of dopamine from the nucleus accumbens and prefrontal cortex (French et al., 1997; Gessa et al., 1998), dopamine receptor 2 gene (DRD2) has been considered a potential candidate for susceptibility to cannabis dependence. For example, the synonymous SNP C957T, rs6277, seems to affect gene expression, modifying mRNA stability (Duan et al., 2003) and it has been related to relatively low values measuring reward sensitivity (Davis et al., 2008). Studies of striatal D2 receptor (D2R) binding potential reported significant association with baseline of 3 variants of DRD2, included the synonymous SNP rs6277 (Hirvonen et al., 2004). A strong association result between schizophrenia and the C/C genotype of the SNP C957T rs6277 (Lawford et al., 2005).
Ankyrin repeat and kinase domain containing 1 (ANKK1) gene has been reported in association with both alcohol and drug dependence (Yang et al., 2008). Specifically, TaqIA SNP (rs1800497; locus 11q22–q23) is the most studied genetic variant in substance use disorders (Ponce et al. 2009), historically referred to DRD2 genes, belongs to the Ankyrin repeat and kinase domain containing 1 gene (ANKK1), adjacent to the DRD2 on the chromosome 11 of human genome. This nucleotide variation causes a glutamate-to-lysine substitution at the 713 aminoacidic residue in the putative binding domain of ANKK1 (Ma et al., 2015) and it has been previously associated with a reduced D2 receptor density in the brain (Jönsson et al., 1999). Although this was not universally accepted (Neville et al. 2004), Gluskin & Mickey (2016) have recently analyzed all the molecular imaging studies testing weather common genetic variants influence D2 receptor binding potential in humans. Among all, they establish a robust association of ANKK1 Taq1A polymorphism with D2 receptor binding potential in healthy subjects, with a lower binding for the carrier of the minor allele A1 (Lys713).
A1 allele appears to interact with conduct disorder (the behavioral phenotype of impulsivity) and carriers adolescents of the A1 allele, conduct disorder or impulsive behavior, present higher levels of problematic alcohol use than those who were non-carriers (Esposito-Smythers et al., 2009). In a Turkish population the A1 allele is proposed to increase the risk of cannabinoid dependence (Nacak et al., 2012).
Other genes implicated in dopaminergic system could be involved in susceptibility to cannabis use disorders. These include dopamine receptor 4 gene (DRD4), dopamine transporter gene DAT and Catechol-O-Methyltransferase gene COMT, directly involved in the catabolism of dopamine, through the introduction of a methyl group from S-adenosyl methionine to the catecholamine (Axelrod & Tomchick, 1958). COMT enzyme function is particularly important in the prefrontal cortex and in the dopaminergic transmission in the midbrain (Pelayo-Terán et al., 2012) and it is influenced by a common SNP that leads to a substitution of valine with methionine at the position 108/158 on chromosome 22q11. This substitution results in differences in the COMT enzyme activity (Männistö & Kaakkola, 1999). The presence of the valine allele results in high enzymatic activity, whereas the presence of the methionine allele is linked to low enzymatic activity; heterozygosity at Val158Met locus results in an intermediate activity level (Chen

et al., 2004). COMT enzyme activity decreases the extracellular dopamine level, effect caused by many drug of abuse; in this context, the Val158Met SNP can increase the vulnerability to cannabis dependence (Baransel et al., 2008). Observational studies have suggested that this functional polymorphism in the catechol-O-methyltransferase gene (Val158Met) may moderate the psychosis-inducing effect of cannabis, with carriers of the Val allele most sensitive to Delta-9-THC-induced psychotic experiences (Henquet et al., 2006). Two other studies have examined whether the relative risk of developing psychosis following use of cannabis is dependent upon rs4680 variation within COMT: an interaction between cannabis use and the high activity allele at rs4680 increased the risk of psychotic symptoms (Caspi et al. 2005) but this result is in contrast with the founding that low activity COMT variants are related to cannabis use in schizophrenic patients (Costas et a., 2011). More recently, the COMT Val158Met polymorphism has been shown to interact with cannabis use in people with an At Risk Mental State (ARMS), a state that may be early, low level, signs of psychosis (Nieman et al, 2016). The contradictory results may be explained by the fact that the relation between drug use and psychosis susceptibility is probably mediated by several genes in addition to COMT genotypes (Henquet et al., 2008; Zammit et al., 2011). In the Appendix, studied polymorphisms with relative references are reported (Table 1a).

VNTRs studies

Converging evidence across multiple methodologies supports the possibility of a robust relationship between the exon 3 VNTR polymorphism of the dopamine receptor gene DRD4 and the risk of addictive behavior starting with cannabis use (McGeary, 2009). DRD4 is a G-protein coupled receptor, more frequent in the insula, hippocampus, cingulated cortex, entorhinal cortex, and temporal cortex (Lathi et al., 2005), which inhibits adenylyl cyclase with a consequent decrease of cAMP biosynthetic process. Among many SNPs showed by this gene, the most investigated genetic variant in the DRD4 gene is a VNTR polymorphism located in the third exon, resulting in the third intracellular loop of the peptide, a repeated sequence of 48 bp in 2–11 copies (van Tol et al., 1992). The more frequent repeats are 4 and 7 copies with a frequency in European ancestry of 61 and 26%, respectively. This variation has been shown to affect the function of the D4 receptor in vivo (Gorwood et al., 2012). Many studies on this variant regard behavioral traits and substance dependence, as alcoholism (George et al. 1993) and nicotine addiction (Laucht et al. 2005; Vandenbergh et al. 2007). In a sample of primarily cannabis users DRD4L was linked to problematic illicit drug use (Kendler et al., 2008). This result has been recently confirmed in a paper reporting that carriers of DRD4L (long, 7 or more repeats) relative to DRD4S (short, 6 or fewer repeats) homozygotes showed greater frequency of marijuana, alcohol use and hard drug use over the last 6 months (Mallard et al., 2016). Carriers of the DRD4 7-repeat, in addition, seem to use cannabis directly related to parental monitoring (Otten et al., 2013). These results are consistent with the hypothesis for which the 7R+ may intensify risk for problematic tobacco and cannabis use (Olsson et al., 2013)

Dopamine transporter gene (SLC6A3, DAT) encode for dopamine transporter that interrupts dopaminergic activity in the synaptic cleft, through a reuptake mechanism. Vandenbergh and co-workers identified a VNTR polymorphism in the genomic sequence encoding the mRNA untranslated region. In Caucasian- and in African-Americans, this VNTR copy number varies between 3–11, with 90% of the individuals displaying 9 or 10 copies (Vandenbergh et al., 1992). This variation can have functional effects, although it is located in an untranslated region and does not modify aminoacidic sequence (Haddley et al, 2008), altering mRNA structure and consequently DAT expression levels (Fuke et al., 2001); in addition, the DAT VNTR 9R carriers have been found to have higher striatal DAT availability (van Dyck et al., 2005) and

higher striatal DAT expression (van de Giessen et al., 2009) than 10R homozygotes. Several studies have investigated the relation of this gene with addiction, because DAT is the primary biological target of cocaine and many other drugs (Desai et al., 2005). An interesting paper regarding VNTR 3'UTR variation in DAT gene investigates the genotype impact, neutralizing potential confounders, as study typology, samples size, ethnicity, statistical procedures. The 9R/9R homozygous genotype has been shown to confer a general protective effect against risky behaviors, including marijuana use (Guo et al, 2010).

1.5.1.3 Genome-wide studies

Three genomewide linkage analyses on cannabis users have determined regions probably involved in cannabis abuse (Agrawal & Lynskey, 2009). Genome wide linkage analysis is a family based method, establishing whether two genes, or two DNA sequences, are associated or independent. The method assesses the likelihood that two adjacent genes or regions are inherited more often together than alone in the addicted relatives (Rich, 1990). The regions identified include monoglyceride lipase gene (MGLL) in chromosome 3, an enzyme that hydrolyses eCBs (Hopfer et al., 2007), the G-protein coupled receptor 68 gene (GPRC68) (Agrawal et al, 2008) and epidermal growth factor, latrophilin, and 7 transmembrane domain containing 1 gene (ELTD1), a G-protein coupled receptor involved in neuropeptide signaling pathway (Agrawal et al, 2008). Linkage studies have also suggested for future investigation to consider the cannabinoid receptor 1 gene (CNR1). Linkage studies limitation is that complex traits, as substance use disorders, require to identify specific genetic regions involve in disease causation, more than long chromosome traits (Agrawal & Lynskey, 2009).
Genome-wide association study (GWAS) is one of the best approach that involves rapidly scanning markers across the complete sets of DNA, or genomes, of many people to find genetic variations associated with specific diseases. To date, three GWASs of cannabis use phenotypes have been published (Agrawal et al. 2011; Verweij et al., 2013; Minică et al., 2015): due to the small effect sizes of common variants these studies did not identified any genome-wide significant associations (Stringer et al., 2016). One recent GWA study from the International Cannabis Consortium found four significant genes associated with lifetime cannabis use, unfortunately not replicated in the replication samples: NCAM1, coding a neural cell adhesion molecule involved in pituitary growth hormone secretion regulation (Rubinek et al, 2003). NCAM1 is part of the NCAM1–TTC12–ANKK1–DRD2 (NTAD) gene cluster, which is related to neurogenesis and dopaminergic neurotransmission and it has been associated to nicotine dependence (Gelernter et al., 2006) and hypothesized to be associated with other substance use disorder (Stringer et al., 2016). Cell Adhesion Molecule 2 gene (CADM2) belonging to the immunoglobulin superfamily has previously already associated with cannabis use (De Alwis et al., 2014). SCOC gene, encodes a short coiled-coiled domain-containing protein that localizes to the Golgi apparatus, the function is still unknown and no other studies found correlation with SCOC gene variants and addiction (Stringer et al., 2016). The last gene is KCNT2 (Potassium Calcium-Activated Channel Subfamily U Member 1), previously associated to cocaine dependence and heavy opioid use (Gelernter et al., 2014).

1.5.2 Environmental studies

Many people start to use drugs, but only a proportion of them develop addiction, even despite the negative consequences that face up in their life. Evidence show that early stressful life events and traumatic experiences are risks factors to develop addiction and can act as cues that trigger relapse (Cadet, 2016).
Since cannabis use can lead deficits in cognitive functioning, interfering with normal brain development (Squeglia et al., 2009) and also adulthood problems such as dependence, psychosocial dysfunction, delinquency, and progression to other drug use (Lynskey et al., 2003), it is crucial the development of early intervention to prevent cannabis use during adolescence (Stapinsky et al., 2016). An essential step in this strategy is to identify the environmental predictors that trigger cannabis use.
Drug addiction in general can be mediated by traumatic conditions, as emotional abuse and emotional neglecting, low perception of parental care and altered infant-parent attachment, adverse experiences during infancy or adolescence. Among a wide range of childhood maltreatments and stressful life events, main conditions have been identified as risk factors for psychopathology: childhood exposure to physical and sexual abuse (Koss et al., 2003), severity of childhood emotional abuse (Hyman et al., 2006), maladaptive family functioning, including parental substance abuse, criminality, domestic violence, childhood abuse and neglect; parental death and other loss, and parental divorce, with associated economic adversity. Also, social deprivation, living in a disadvantaged neighbourhood is associated with drug addiction, remarking how substance use disorders development are unlikely to result entirely from personal attributes (Kendler et al., 2014). These environmental negative factors have been associated with an increased risk of physical and emotional health outcomes, psychopathology, alcoholism, and drug dependence (Enoch, 2011). They could alter reward, emotion processing and decision making control systems, creating an ideal background for addiction development. How environmental conditions affect these pathways and complex systems can be explained through different mechanisms.

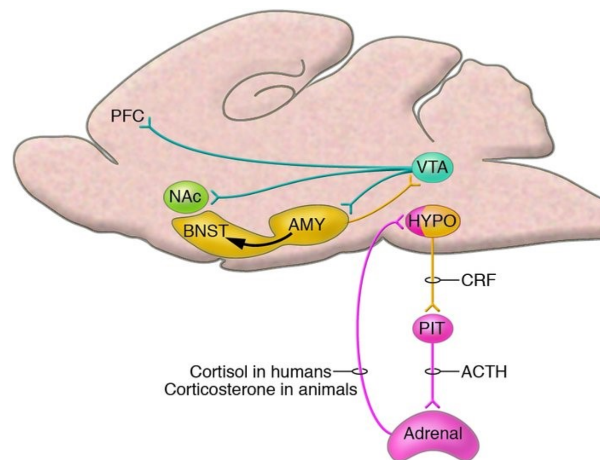


Figure 10. Reward and stress pathways in the brain. The mesolimbic dopamine reward pathway (blue). Central CRF circuitry corticotropin-releasing factor (CRF) system (yellow) (Cleck et al., 2008).

First, stress can affect neuronal plasticity, particularly in the mesolimbic dopamine pathway that is fundamental to the drug-induced sensation of pleasure that acts as positive reinforcement (Sapolsky 2003). The reward pathway originates in the VTA of the midbrain and projects to the NAc, the limbic system, and the orbitofrontal cortex. The amygdala, hippocampus, and medial prefrontal cortex send excitatory projections to the NAc (Figure 10) (Cleck et al., 2008). Alcohol and drug intake is associated with increased synaptic DA in the NAc and elsewhere in the reward pathway.
Although the activation of the hypothalamic–pituitary–adrenal (HPA) axis and catecholamine systems in response to acute stress are essential for survival, chronic activation results in increased risk for numerous physiological conditions as well as vulnerability to psychopathology such as anxiety, depression, alcohol and drugs use disorders (Sapolsky et al. 2000). Stress may also affect addiction vulnerability influencing

gene transcription in the brain and neuroendocrine system. For example, acute stressors were shown to alter hippocampal gene expression (Gray et al, 2014). Severe stress seems to lead to measurable brain damages, with alterations of hippocampal, amygdala and prefrontal cortex volume (McEwen, 2006). Supporting evidence to all these observations come also from animal studies (Enoch, 2011).
Finally, more recently evidence suggest that both acute and chronic stress can impact epigenetics changes (Chakravarty et al., 2014): child abuse, low maternal care perception, paternal deprivation can cause long lasting epigenetic changes in adult mammals (Cadet et al., 2016). For example, prenatal exposure of maternal depression was associated with increase methylation of the neural specific glucocorticoid receptor (NR3C1) (Oberlander, 2008); the same result has been documented in suicide victims with child abuse history (McGowan et al., 2009).
Focusing on cannabis use disorder, vulnerability to both cannabis use initiation and problematic has been shown to be affect by genetic and environmental factors in a twin study, but with a greater contribution of the environment, rather than genetic components, for female (Verweij et al., 2010).
Cannabis and alcohol was strongly associated with externalizing problems, as attention deficit hyperactivity disorder (ADHD) (Heron et al., 2013), troublemaker at school, conflict with teacher, disruptive classroom, expelled from school and violent behavior (Pedersan et al., 2016). In this context, peers influence appears important so much that a study considers the number of peers using cannabis as the main predictor of cannabis use. Also, parental influence exerts a great effect during the preadolescent years on substance use (Chabrol et al., 2006).
Several studies provide evidence that childhood maltreatment, childhood abuse, sexual abuse, exposure to community violence and other early life traumatic events, as severe negative life events may influence cannabis consumption (Harrison et al., 1997; Kilpatrick et al., 2000). Subjects who developed full and partial Posttraumatic Stress Disorder (PTSD), following a range of community and family-based traumas, witness homicide, threatened with a weapon, sexual and physical abuse, reported greater cannabis use (Lipschitz et al. 2003).
Hyman and Sinha discuss childhood maltreatment along with other types of impacts on cannabis consumption. They highlight the urgency to become more aware of children and adolescents' stressors and implement strategies to prevent cannabis use, not only for undoubtedly stressful experiences as childhood abuse, but also for devious and less obvious condition, as the anxiety manifested in social pressure (Hyman & Sinha, 2009).
Since substance use disorder is a multifactorial complex neuropsychiatric disorder, adverse environment conditions may confer sensitivity to increased psychiatric illness and drug addiction. These can also impact multiple genetic markers. On the other hand, family, community involvement and individual resilient factors may also protect these individuals against drug addiction effects and improve the treatment of patients or teach to those who are living in high-risk situations (Cadet et al., 2016).

1.5.3 Epigenetic studies

1.5.3.1 Overview of Epigenetics

Interaction between genotype and environmental factors can influence drugs of abuse response and dependence through epigenetic mechanisms (Nielsen et al., 2012).
Epigenetics refers to heritable changes that are not the result of modified DNA sequence (Tchurikov, 2005) and includes alteration in the accessibility of DNA, potential changes in translational processes, meiotically and mitotically inherited transcriptional alterations (Kota & Feil, 2010). If we consider the genetic code as a sentence, epigenetics acts through changes that can profoundly alter the meaning of the sentence itself. These changes provide the genome with a high degree of flexibility in terms of transcriptional output and cellular phenotype (Paluch et al., 2016).

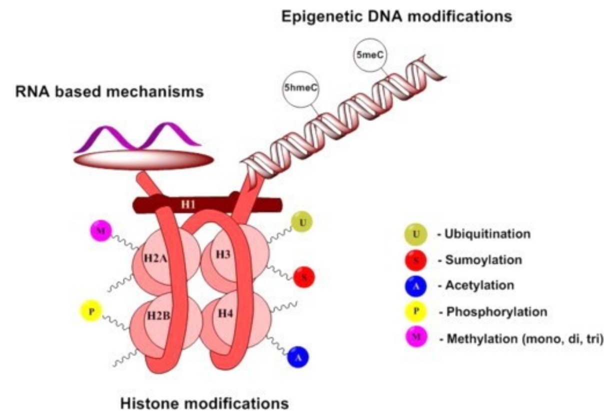


Figure 11. Overview of epigenetic processes taking place in a cell. Chromatin organization: DNA is wrapped around histones H2A, H2B, H3 and H4 (pink) to form nucleosomes. DNA modifications and post-translational modifications of the histone tails, which protrude from nucleosomes, are shown (Mikhed et al., 2015).

The DNA in the nucleus of all eukaryotic cells is organized into chromatin (Figure 11). The basic unit of chromatin is the nucleosome, an octamer that consists of two copies each of H2A, H2B, H3, H4 protein histones surrounded by 147 base pairs of DNA wrapped approximately 1.7 times around this complex. Epigenetic mechanisms control the degree to which nucleosomes are condensed, which affects gene activity. Subsequently, chromatin exists in many states between two extremes: an inactivated, condensed state, heterochromatin, which does not allow transcription of genes, and in an activated, open state, euchromatin, which allows individual genes to be transcribed. Complex biochemical processes regulate the state of chromatin, in genic or non-genic regions, involving DNA methylation, DNA hydroxymethylation, histone modifications and transcriptional and posttranscriptional changes mediated by noncoding RNAs (Nestler et al., 2014).

DNA methylation

DNA methylation is relatively stable mark characterized by a covalent modification at the 5-position of cytosine to form 5-methyl cytosine (5-mC) (Figure 12). The reaction is mediated by DNA methyltransferases (DNMT) (Robertson et al., 2005). To date it has been discovered the existence of a maintenance DNA methylation occurring during DNA replication, predominantly dependent on DNMT1, that recognizes hemimethylated DNA and carefully copies the DNA meth pattern from parents to daughter strand, whereas de novo DNA methylation is carried out by DNMT3A, DNMT3B, and DNMT3L and establishes the DNA methylation patterns in the germ line or early in embryo (Okano et al., 1999; Kareta et al., 2006). DNA methylation is generally associated to repression of gene transcription through recruitment of co-repressor complexes (Robinson & Nestler, 2011).

Cocaine affects the expression of methyl-CpG binding protein 2 (MeCP2), which regulates BDNF expression (Im et al., 2010): cocaine self-administration is decreased in MeCP2 knockdown rats and seems to increase MeCP2 phosphorylation in the NAc by regulating BDNF expression. Recently, cocaine self-administration was also reported to increase MeCP2 phosphorylation in NAc and this phosphorylation was involved in limiting cocaine intake (Deng et al., 2014). Consistent results are reported from experiments with amphetamine and methamphetamine administration that show how both these psychostimulants impact MeCP2 expression in the brain (Deng et al., 2010; Jayanthi et al., 2014). These changes correlate also with an increased expression of DNMT1 in the brain (Numachi et al., 2007; Jayanthi et al., 2014).

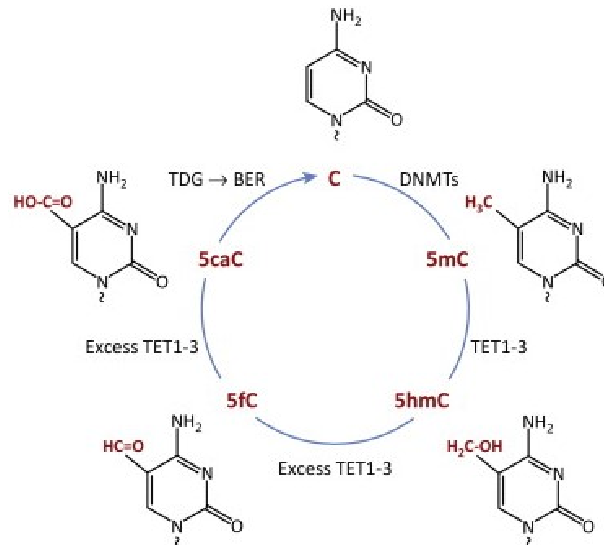


Figure 12. Cytosine modification cycle. Cytosine is converted into 5-mC from DNMT in a reaction mediated by DNA methyltransferases (DNMTs). 5mC can be after oxidized to 5hmc by the TET1, TET2, and TET3 enzymes. Excessive TET activity can promote further oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Thymine-DNA glycosylase (TDG) and the base excision repair (BER) can than yield unmodified cytosine (Ivanov et al., 2014).

More than 400 CpGs show differences in DNA methylation between alcohol-dependent cases and controls. Many of these hypermethylated genes are involved in the neurobiology of reward response and were previously associated with alcohol use disorder, including dopamine receptor D4 (D4R), dopamine beta hydroxylase (DBH), dopamine transporter (SLC6A3), cytochrome P450 2E1(CYP2E1), and Toll-like receptor 4 (TLR4). Interestingly, significant correlation was observed across tissue types (Hagerty et al., 2016).

DNA hydroxymethylation

During the process of demethylation, the addition of a hydroxyl group to 5-mC forms the intermediate 5-hydroxymethylcytosine (5-hmC), this reaction is catalysed by the ten-eleven translocation (TET) enzymes, abundant in the brain (Kriaucionis & Heintz, 2009). DNA hydroxymethylation occurs mainly at gene promoter site and this passive-active demethylation it is more correlated with transactivation (Szulwach et al., 2011). Repeated cocaine administration in mouse induces 5hmC, correlated with a downregulation of TET1 in mouse NAc (Feng et al., 2015). DNA hydroxymethylation might have an important role in abstinence from drug-taking behaviors: a recent studied in a rat self-administration model of methamphetamine shows differential patterns of 5-hmc in addicted rats in comparison with nonaddicted rats especially at intergenic sites located on long and short interspersed elements. The same authors report differential 5hmc levels in genes encoding voltage and calcium-gated potassium channels in the NAc, together with an increase in mRNA levels of these potassium channels. This observation opens a possible treatment approach for methamphetamine addiction, since potassium signaling may have a role in regulating abstinence and compulsive drug taking (Cadet et al., 2016).

Histone modifications

Histone modifications are post-translational modification found on histone tails mediated by specific enzymes (Figure 13). The most studied modifications are acetylation that generally promotes decondensation of chromatin and increases gene activity by negating the positive charge of lysine residues in histone tails and increases spacing between nucleosomes. Another important modification is histone methylation that can either promote or repress gene activity, depending on the aminoacidic residue undergoing methylation. Other histone modifications that can impact gene expression include phosphorylation, sumoylation and ubiquitylation (Kouzarides, 2007). These changes can alter gene expression by modifying chromatin conformation and allowing or inhibiting recruitment of regulatory factors onto DNA sequences (Maze & Nestler, 2011). The diversity of histone modifications supports the "histone code hypothesis," which says that the sum of modifications at a particular gene defines a specific epigenetic state of gene activation or silencing.

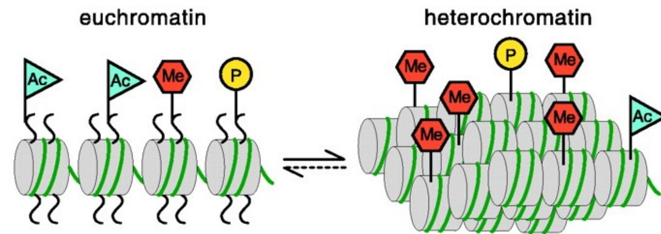


Figure 13. The histone code hypothesis: a model for euchromatic or heterochromatic histone tail modifications (Jenuwein & Allis 2001).

Several studies show how stressful conditions, as paternal and maternal deprivation, history of child abuse and depression, affect the epigenome during adulthood, adolescence and at prenatal level (Cadet JL, 2016). Epigenetic modifications have also a key role in the neuroplastic changes that can lead to addiction phenotype (Hyman et al., 2006; Kalivas et al., 2005; Koob and Kreek, 2007; Levine et al., 2005; Cadet et al., 2016), affecting gene expression and human behaviour. Epigenetic changes have been found to occur in response to illicit drug use, especially cocaine, alcohol and heroin.

Many researches investigate which factors, downstream of histone modifications, play a role mediating the drug seeking behavior in rodents. A major role in the regulation of addiction has been attributed to immediate early genes, such as *c-Fos, c-Jun,* and *FosB*, and transcription factors and coactivators, as cAMP response element-binding protein (CREB) and CREB-binding protein (CBP), protein kinases A and C and other proteins, such as Ras, in the plasticity induced during learning or drug use (Levenson et al., 2004; Shalin et al., 2006; Hitchcock et al., 2014). The transcription factor ΔFosB, in particular, is induced several folds in the NAc by chronic drug exposure and has been implicated in the transition to an addicted state (McClung & Nestler, 2003). A study shows how methamphetamine induces changes in gene expression by epigenetic mechanism, especially increased of phosphorylated CREB (Krasnova et al., 2013).

Multiple drugs of abuse induce changes in histone acetylation (Robinson & Nestler, 2011). A genome-wide analysis of acetylated histones H3 and H4 in cocaine addiction reveals increased binding of acetylated H3 and H4 at the promoters of sirtuins gene (Sirt1 and Sirt2), which are induced in the nucleus accumbens by cocaine (Renthal et al., 2009). Sirtuins, belonging to histone deacetylases class III (de Ruijter et al., 2003). At behavioural level, altered histone acetylation has been found to affect behavioural sensitivity to cocaine, with the inhibition of histone deacetylase being able to potentiate the rewarding effects of the drug at the place preference test (Itzhak et al., 2013).

A significant increase in deacetylases protein expression (HDAC1, HDAC2, SIRT1 and SIRT2) in a rat model has been found after chronic methamphetamine (Jayanthi et al., 2014) and even a single dose of

methamphetamine (20mg/kg) induced expression changes in the HDAC (Martin et al., 2012). In addition enrichment of acetylated histone H4 on glutamate striatal receptors promoters seems to represent the determinant repressive factor in the striatal glutamate receptor expression induced by methamphetamine (Jayanthi et al., 2014).

Drugs of abuse also directly regulate histone methylation: cocaine dramatically alters histone H3 lysine 9 trimethylation (H3K9me3) in the NAc decreased H3K9me3 enrichment at specific genomic sequences, long interspersed nuclear element (LINE) (Maze et al., 2011). Chronic morphine has been associated with the same effect: decrease in G9a expression, a histone methyltransferase that catalyses the euchromatic dimethylation, and global levels of H3K9me2, in mouse NAc (Sun et al., 2012). Di and trimethylation of H3K4, with the associated epigenetic writers and erasers, seem to upregulate transcription of the oxytocin receptor and Fos protein in the NAc, mediating methamphetamine-associated memory development, that increase the relapse vulnerability to substance use disorder by triggering craving (Aguilar-Valles et al, 2014).

Non-coding RNA

More recent studies investigated the epigenetic mechanism mediated by ncRNAs (Figure 14). Large number of expressed RNAs is not translated into proteins and they play crucial regulatory roles in cell function (Taft et al., 2010). microRNA (miRNA), for example, can regulate gene transcription by binding targeted mRNA and modify the state of chromatin by direct interaction with transcription factors and other nuclear proteins that are part of chromatin modifying complexes (Pietrzykowski, 2010). miR-212, miR-181a and miR-124 for example seem to have an important role in cocaine addiction through the CREB–BDNF pathway (Hollander et al., 2010). Argonaut 2 protein (AGO2) that plays an important role in microRNA-mediated gene silencing is involved in cocaine-mediated regulation of gene expression (Schaefer et al., 2010). Other microRNAs have also been shown to affect the expression of the dopamine transporter (Chandrasekar & Dreyer, 2009) or to be regulated by opioids (Barbierato et al., 2015). miR-9 probably influences alcohol reward targeting the D2 dopamine receptor (Pietrzykowski, 2010).

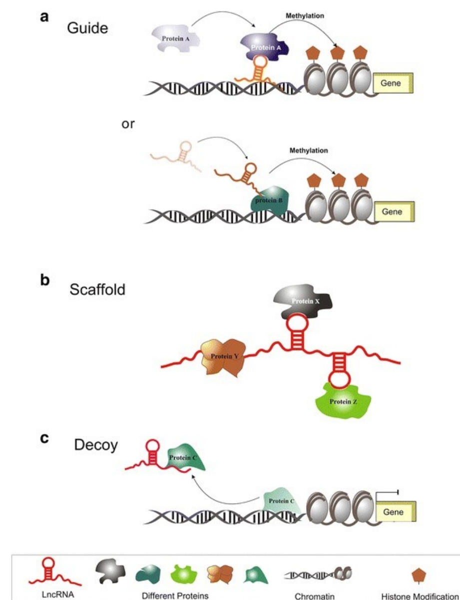


Figure 14. LncRNA hypothesized mechanism of action; lncRNA can (a) interact with a protein to target genomic loci or (b) form complexes or (c) draw proteins away from t target genes (Yang et al., 2015).

1.5.3.2 Epigenetic alterations of marijuana exposure

Cannabinoid exposure has been shown to modulate endocannabinoid-associated pathways through epigenetic modifications (Szutorisza & Hurd, 2016). Long-term cannabinoid exposure has been explored using animal models and they focus especially on the NAc, a critical brain reward area for addiction development (Koob & Volkow, 2010). Prenatal THC exposure can affect offspring behavior (Singh et al., 2006) and brings neurofunctional deficits in the adult offspring rats. These behavioral abnormalities were also associated with changes in the expression of genes related to glutamatergic and noradrenergic systems (Campolongo et al., 2007). Another study investigated the sensitivity of the opioid neuropeptide proenkephalin (PENK) to THC exposure in an adolescent THC-exposed rat model: the normal pattern of H3K9 methylation results altered with a decrease in histone H3 lysine 9 (H3K9) methylation in the NAc shell. In addition, overexpression of PENK potentiated heroin self-administration in the same animals (Tomasiewicz et al., 2012). The fact that adult offspring of THC-exposed parents showed an increase in heroin self-administration, in association with mRNA expression alteration of cannabinoid, dopamine, and glutamatergic receptor genes and epigenetic abnormalities (Szutorisz et al., 2014), seems to support the hypothesis that some epigenetic alterations might be inherited through the germline from parent to child (Bohacek & Mansuy., 2013).

A drug-related cross-generational epigenetic effect has been confirmed comparing DNA methylation in 16 rats with parental THC exposure and 16 without, finding 1027 differentially methylated regions (Watson et al., 2015). This study identified DNA methylation alterations located in the DLG4 /PSD95, gene encoding the postsynaptic density protein 95, a membrane protein involved in the organization of neurotransmitter receptors in the synaptic cleft. It also heteromultimerizes with another protein, DLG2, and is recruited into NMDA receptor and potassium channel clusters.

Richardson and colleagues (2016) hypothesized the prenatal THC exposure as a "first hit" to eCB system, referring to the disruption of eCB system modulation in early development: passed the maternal bloodstream, THC crosses the placenta (Grotenhermen, 2003), leading human fetal distress and growth retardation (Day & Richardson, 1991). Emotional control issues, cognitive impairment, or depression shown by THC-exposed offspring (Jutras-Aswad et al., 2009) might contribute to the higher drug addiction vulnerability and act as the "second hit" postnatally to the eCB system (Richardson et al., 2016). These issues might be explained through the action of epigenetic mechanism: maternal cannabis use in human has been associated with alteration of mesolimbic D2R, a decreased DRD2 expression accompanied by reduced D2R binding sites (DiNieri et al, 2011). As mentioned earlier histone methylation, depending on the aminoacidic residue undergoing methylation, has antagonistic effects on gene activity. Offspring of pregnant rats exposed to THC show increased 2meH3K9 repressive mark, decreased 3meH3K4 and RNA polymerase II association with the promoter at the DRD2 locus in the NAc. Furthermore, reduction of D2R seems to increase the addiction vulnerability later in life, because THC-exposed offspring showed an increased sensitivity to opiate reward in adulthood (DiNieri et al., 2011).

Even though the relatively easy accessibility and frequent use and abuse of marijuana, very few studies have been published about epigenetic effects associated with cannabis exposure in human.

Some of these studies have investigated the relation between endocannabinoid system and epigenetic changes in different pathological conditions, showing, for example, increased CB1 receptor expression in peripheral blood lymphocytes of schizophrenic patients with cannabis abuse, inversely correlated to methylation of the CNR1 promoter (Liu et al, 2014). In another study, CB1 gene expression and DNA

methylation of its promoter were found to be altered in peripheral blood cells of subjects with THC dependence (Rotter et al., 2013).
Recently, a “trials study”, regarding the gene-environment epigenetic associations, showed adolescents with the Met/Met genotype of COMT gene in combination with high rates of COMT promoter methylation is less likely to be high-frequent cannabis users when compared to adolescents with the Val/Val or Val/Met genotype (van der Knaap et al., 2014).
It is evident that a complex relationship exists between genetic and epigenetic interactions, and even more complex interplay between peripheral epigenetic marks and methylation status in the brain (Szutorisza and Hurd, 2016). These findings suggest epigenetic regulation in peripheral blood lymphocytes as an easily accessible biological marker for the study of THC action and dependence (D’Addario et al., 2013).

1.6 Blood and brain interplay in the biomarkers research perspective

Epigenetic changes are often tissue- and cell- specific, and usually more located in the brain regions where addiction conspicuously acts. For this reason, epigenetics is studied especially in post-mortem brain tissue, but unfortunately high-quality post-mortem brain samples are a limited resource (Wong et al., 2011), that can even be subjected to artefacts, due to pharmacological treatments, cause of death and post-mortem interval length (Li et al., 2004; Hashimoto et al., 2007). It is therefore complex to determine whether the observed changes are compensatory effect due to the continued disease, its treatment or to other factors (Hayashi-Takagi et al., 2014). To investigate how epigenetic modifications found in more accessible tissue, as peripheral blood, might serve as alternative markers for brain tissue is an important issue to consider if we are to develop rational treatment for addicts (Walton et al., 2015). To date many studies on epigenetic changes in neurological disorders, such as autism, schizophrenia, Alzheimer’s, Huntington’s, Parkinson (Urdinguio et al., 2009), Major depressive disorder (Numata et al., 2015), cannabis addiction (Rotter et al., 2013), have revealed peripheral blood as a potential source of biomarkers that may reflect the state of illness in the brain (Tylee et al., 2013).
Even if not all the epigenetic changes of peripheral blood cells are similarly reflected in the brain, a close correspondence may indeed exist for many genes (Davies et al., 2012; Masliah et al., 2013; Aberg et al., 2013; Tylee et al., 2013). For example, peripheral lymphocytes, the potential source of genomics DNA, express OPRM1 gene, encoding the mu opioid receptor, which is the primary site of action for the most commonly used opioids (McCarthy et al, 2001) and also several genes involved in dopaminergic neurotransmission that play a crucial role in the genesis and maintenance of drug addiction (Kordi-Tamandani et al, 2013; Hillemacher et al, 2009; Hayashi-Takagi et al., 2014). In this perspective, CNR1 DNA methylation and CNR1 mRNA expression, found altered in peripheral blood cells of subjects with THC dependence, can be considered as potential peripheral biological markers (Rotter et al., 2013).
Thus, the possibility to study epigenetic changes in peripheral tissue opens new challenges in the identification and development of biomarker (Tylee et al., 2013) associated to drug addiction and its treatment, underling the importance to conduct more studies to establish the functional relevance of peripheral epigenetic changes on neurobiological alterations induced by drug use (Szutorisz & Hurd, 2016).
All these observations emphasise the role of epigenetics as a biological mechanism for environmental exposure associated with human diseases that may influence processes that lead to diseases or may serve as biomarkers of potential environmental exposure (Ladd-Acosta, 2015).

2. Aim of the project

Cannabis is the most commonly used illicit drug globally. As outlined in the introduction section, Cannabis use is associated with dependence, craving, and drug-seeking behavior. The drug has a wide range of psychological and physical effects.

Because co-occurrence of genetic, environmental, and epigenetics factors that may increase the susceptibility to cannabis use disorder, to identify all these probably causes of addiction, it is very important to understand the neurobiological bases of marijuana abuse in order to develop better therapeutic and preventive approaches to the disorder.

No medication has been shown broadly effective in the treatment of cannabis dependence, nor is any medication approved for this condition (Danovitch and Gorelick, 2012). The end goal of this project is meant to realize prevention programs and strategies for treatment of cannabis use disorders enhancing protective factors, that reduced potential for drug use and reducing risk factors, which make drug use more likely.

Aim 1

To verify the potential role of gene polymorphisms in the development of cannabis use disorders, comparing a group of marijuana users and a group of healthy control subjects.

Neurotransmitters and their receptors play a key role in drug addiction. Susceptibility to drug dependence can be affected by genetic variants in genes involved in drugs of abuse metabolism or in the dopaminergic pathway and the endocannabinoid system.

Aim 2

To investigate the role of environmental factors in the susceptibility of cannabis use disorders and the possible interaction of adverse experiences with genetic factors in the etiology of this disorder.

Drug addiction is a multifactorial condition, where genes and environment interact with each other's. Environment stimuli might modify the genetic risk to cannabis use disorders, such as early childhood adversity or stressful life events.

Aim3

To quantify DNA methylation in specific sites of genes involved in the neurobiology of cannabis use disorders, comparing marijuana users and control subjects.

Epigenetic modifications can affect gene expression and human behavior. Understanding which epigenetic changes could mediate drug addiction or could act as susceptibility factors in its development is a new promising research area to treatment improvement.

3. Methods

Two studies were conducted, one in Caucasian population and the second one in American population. The workflow below shows the steps included in the projects (Figure 15). The first (1) phase was, for both population, human samples and environmental data collection (environmental data were represented by psychometric tests in Caucasian subjects and by demographic data in American population). The second phase (2) was gDNA extraction, followed by genotyping (3); two different genotyping strategies has been used: a gel electrophoretic based analysis for Caucasian study and TaqMan genotyping Assay (Thermo) for the American study. After the genotyping step, two logistic regression models (4) evaluated, first, the influence on environmental factors on marijuana use and, second, the combined influence of genotypes and environmental factors on marijuana use. Finally, DNA methylation analysis through MeDIP-qPCR has been carried out on American population.

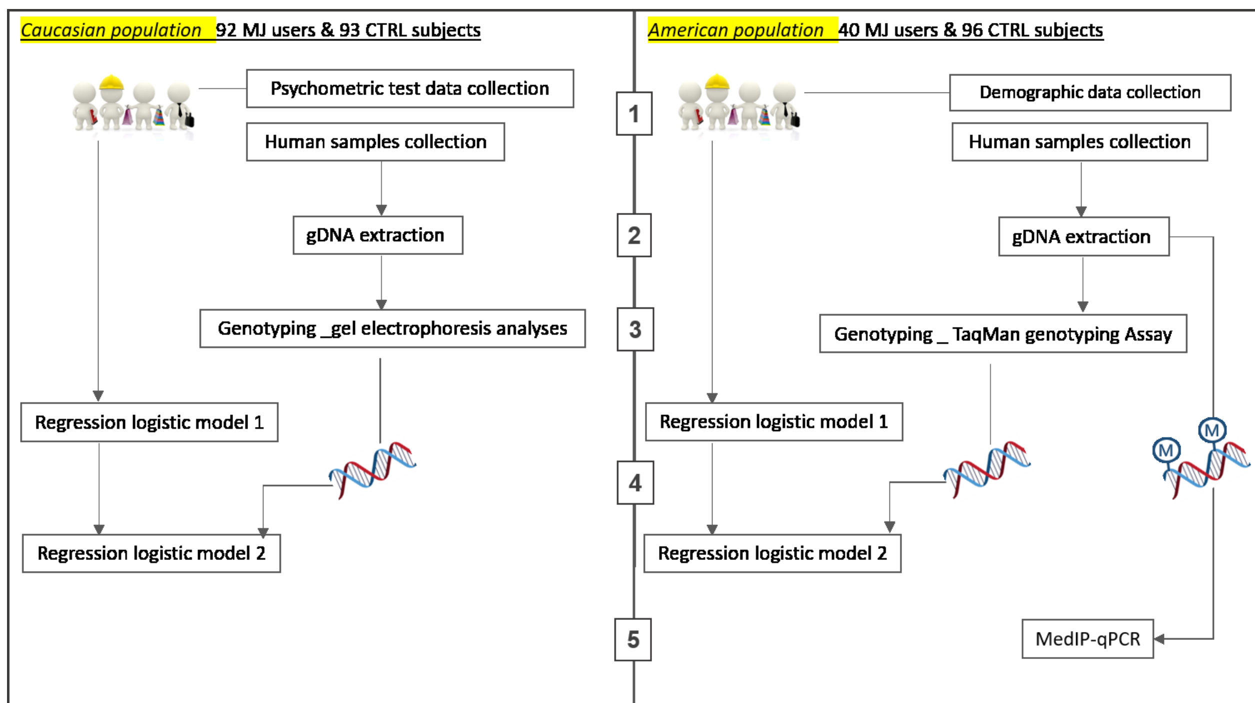


Figure 15. Workflow diagram on Caucasian and American population.

3.1 GENETIC STUDY

3.1.1 Participants

Caucasian population

The 92 marijuana users included in the current analysis met the following criteria:

- Regular adult caucasian smokers of marijuana, daily or near daily cannabis users, who got in touch with treatment services of the public health system (most of them approached the services because of the law imposing to drug users to have at least a contact with treatment service and to provide negative urines for 3 months; others were treatment seeking for behavioural problems induced by cannabis).
- All of them provided positive urines for cannabis and negative for all the other drugs at the beginning
- Serious mental health disorders pre-existing to cannabis use disorder were excluded.

93 unrelated healthy individuals from the same geographical areas were selected as healthy volunteers. They were recruited as volunteers who have never used illicit psychotropic drugs, from hospital and university staff workers, blood donors and university students.

Having severe somatic disorders (chronic liver or renal disorder, endocrinopathies, immunopathies and HIV disease) and other psychiatric diseases represented an exclusion criterion.

Each subject was also submitted to three psychometric tests: ACES, CECA-q, PBI.

American population

Forty (40) marijuana users and ninety-six (96) control subjects were selected among samples collected and stored previously at the Intramural Program of the National Institute of Drug Abuse (NIDA). All these subjects were recruited among people who live in Baltimore City or one of the surrounding counties.
The eligibility criteria to select marijuana users' samples was to use marijuana ten times or more during the past thirty days and no other drug use.
For each subject, demographic and vital data were also collected.

For both the studies were included 18-60 years old subjects.
The participants were also informed about the experimental procedures and the study aim before giving their written consents. Each volunteer (cases and controls) signed an informed consent document before entering the study.

3.1.2 Polymorphisms selection

The candidate gene polymorphisms were selected for the study, because involved in dopaminergic and endocannabinoids pathways. Furthermore, based on literature analysis (See Appendix, Table 1a), previous findings identified them as attractive candidates in the discovery of drug addiction risk factors. Indeed, they could be interesting source of variations for cannabis use disorders (Table 1).

	Gene	SNP/VNTR	DNA sequence variation	Position	Functional Consequence	Global MAF
Caucasian population	CNR1	rs1049353	A/G (REV)	6:88143916	synonymous codon: Thr ⇒ Thr	T=0.1294/648
	FAAH	rs324420	A/C (FWD)	1:46405089	missense: Pro ⇒ Thr	A=0.2616/1310
	COMT	rs4680	A/G (FWD)	22:19963748	missense, upstream variant 2KB: Val ⇒ Met	A=0.3692/1849
	DRD2	rs6277	C/T (REV)*	11:113412737	synonymous codon: Pro ⇒ Pro	A=0.2440/1222
	ANKK1	rs1800497	C/T (REV)	11:113400106	missense: Glu⇒ Lys	A=0.3257/1631
	DAT1	VNTR 3'UTR	40bp, 3-11 repeats	5p15.3; 3'UTR	3'UTR	/
	DRD4	VNTR exon 3	48bp, 2-11 repeats	11p15.5; exon3	exon 3	/
American population	ANKK1	rs1800497	C/T (REV)	11:113400106	missense: Glu⇒ Lys	A=0.3257/1631
	CNR1	rs1049353	A/G (REV)	6:88143916	synonymous codon: Thr ⇒ Thr	T=0.1294/648
	CNR1	rs2180619	A/G (FWD)	6:88168233	upstream variant 2KB	G=0.4685/2346
	CNR1	rs806379	A/T (FWD)	6:88151548	intron variant, upstream variant 2KB	T=0.3952/1979
	CNR1	rs6454674	G/T (FWD)	6:88163211	intron variant	G=0.3141/1573
	CNR1	rs12720071	A/G (REV)	6:88141462	UTR variant 3'	C=0.0899/450
	CNR1	rs2023239	C/T (FWD)	6:88150763	intron variant, upstream variant 2KB	C=0.1779/891
	CNR2	rs2501431	A/G (FWD)	1:23875153	synonymous codon: Gly ⇒ Gly	G=0.3466/1736

Table 1. List of candidate genes, relative functions and analysed polymorphisms in the Caucasian and American studies. Notes: (*) rs6277 C957T SNP, has been studied in association with the mutation G1101A. DNA sequence variation refers for the SNPs to RefSNP Alleles.

3.1.3 Genotyping method 1

Caucasian population

The genotyping procedure in Caucasian population was carried out in 4 main steps:

3.1.3.1 Biological samples collection

Blood or saliva samples were collected. FTA (Fast Technology for Analysis of nucleic acids) classic cards (Whatman) have been used for blood collection; buccal swab (Whatman) for oral mucosa collection. FTA classic cards were stored and subsequently processed for DNA extraction. The buccal swabs were immediately subjected to the DNA extraction protocol (Figure 16).

Figure 16. (a) FTA Classic Card; (b) Buccal swab (Whatman).

3.1.3.2 Genomic DNA extraction

DNA extraction from buccal swab

DNA extraction from the buccal cells, collected using buccal swabs, was carried out using QIAamp® DNA Mini Kit (Figure 17). A small quantity human buccal cells were obtained with an oral swab with a soft bristled, sterile cytobrush. The buccal swab was dislodged into a 2mL tube, subjected to lysis buffer treatment and to further protein digestion. The resulted solution is then added to silica-gel membrane spin column (QIAamp Mini spin column) that selectively bind DNA based on pH and salt concentrations. Genomic DNA (gDNA) is eluted with TE Buffer (10 mM Tris-Cl, 0.10 mM EDTA; pH 8.0) or AE buffer, after the spin column matrix is washed to remove proteins and other impurities that can inhibit PCR and other downstream enzymatic reactions.
The concentration of the isolated gDNA and its purity was determined using NanoDrop 2000c Spectrophotometer (Thermo Scientific).

Figure 17. DNA extraction from buccal swab, using QIAamp Mini spin column.

Purification of FTA Classic Cards from blood samples

FTA classic cards guarantee a protection of nucleic acids from degradation at room temperature.
After sample application on the card and allowed to dry it completely, 2 disks were punch out of the sample area on the FTA Card (Figure 19). The disks were placed in PCR tube and washed three times with FTA Purification Reagent. Two more washes with TE-1 buffer (10 mM Tris-Cl, 0.10 mM EDTA; pH 8.0) were conducted. The DNA got entrapped within the FTA Matrix (Figure 18).
The disks were dried in PCR tube and PCR master mix directly added to the disks and amplified.

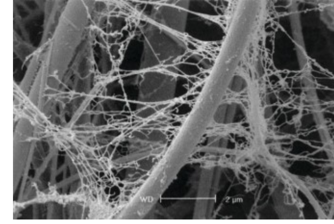


Figure 18. Electron micrograph showing DNA in the FTA matrix (10,000× magnification)

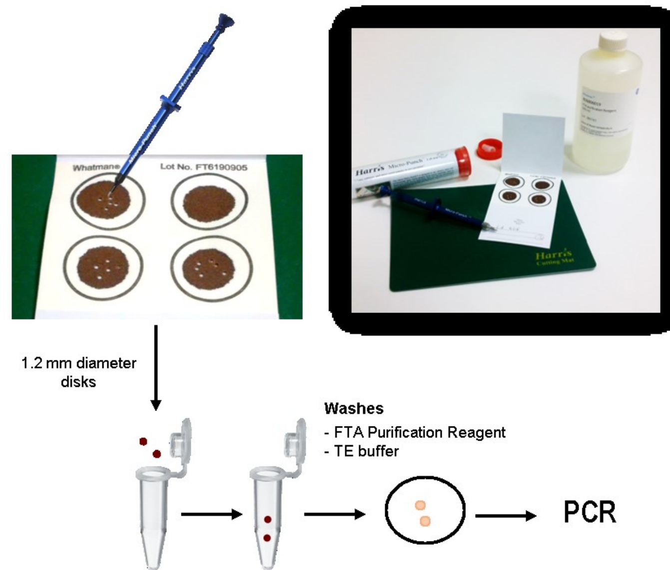


Figure 19. FTA Classic Cards (Whatman) Purification Protocol.

3.1.3.3 Amplification of the polymorphic regions

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification (Saiki et al., 1985). Taq DNA Polymerase is an enzyme widely used in PCR, isolated from Thermus aquaticus YT1 (Chien et al., 1976). The enzyme consists of a single polypeptide with a molecular weight of approximately 94 kDa. Taq DNA polymerase is heat-stable (no significant loss of activity at 95 °C) and will amplified DNA at elevated temperatures from single-stranded templates in the presence of the primers. It has 5'→3' DNA polymerase activity and 5'→3' exonuclease activity. Platinum™ Taq DNA Polymerase (Invitrogen) has been used: a hot-start enzyme provided in an inactive form, due to specific binding of the inhibitor. PCR is activated in a temperature-dependent manner (at 94ºC), regaining its full activity. For most of the genes the following standard PCR protocol was applied, using human oligonucleotide primers previously selected (See Table 3). The master mix was assembled with the components listed in Table 2

and incubated with the samples in a thermal cycler at 94°C for 2 minutes to completely denature the template. After 35 cycles of PCR amplification (denaturing 94°C for 30 s, annealing 55°C for 30 s, extension 72°C for 30 s) the samples were incubated for an additional 7 min at 72°C and maintained the reaction at 4°C (Figure 20). The samples were then stored at –20°C until use.

Component	Volume	Final concentration
gDNA	10-100 ng*	0,4 - 4 ng/µl
10X PCR buffer	2.5 µl	1X
50 mM $MgCl_2$	0.750 µl	1.5 mM
10 mM dNTPs mixture (dATP, dTTP, dGTP, dCTP)	0.5 µl	0.2 mM each
Primer Forward (10 µM)	0.5 µl	0,2 µM
Primer Reverse (10 µM)	0.5 µl	0,2 µM
Platinum Taq DNA Polymerase Invitrogen (5 U/µl)	0.1 µl	0,02 U/µl (1 U tot)
Autoclaved distilled water	to 25 µl	n/a

*2 µl gDNA extracted with QIAamp DNA Mini Kit (QIAGEN).
2 disks from 1.2 mm FTA Card (Whatman).

Table 2. PCR component for standard protocol.

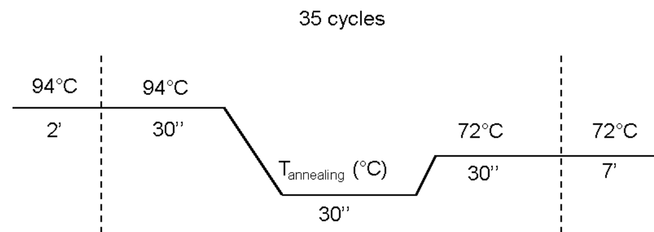


Figure 20. PCR amplification condition for standard protocol.

To amplify the SNP regions belonging to DRD4 and CNR1 genes further optimization of the standard protocol were required, since these regions are templates with high GC content and high secondary structure. To avoid nonspecific amplicons, touchdown PCR in combination with additional denaturing agent (10% DMSO), to the basic components of the standard protocol, was performed. During the touchdown PCR, the annealing temperature is gradually reduced (0.5°C /every cycle) (Figure 21).

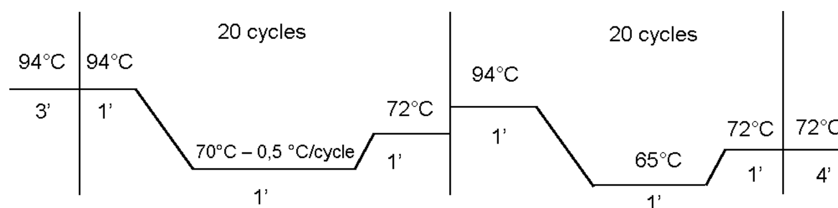


Figure 21. CNR1and DRD4 PCR-amplification conditions.

3.1.3.4 Identification of allelic variants using gel electrophoresis

Allelic variants identification was different according to polymorphism typology.
In case of length polymorphism (VNTR), after PCR reaction, DNA amplicons were directly loaded on agarose gel electrophoresis. The amplicons length varies depending on the number of repeats of that specific allele. The DNA molecules were visualized under UV light on UV-Transilluminator (agarose gel was previously prepared with Red Safe). Proper DNA size marker was always loaded along with experimental samples. Amplicon length, and therefore the number of repeats within the amplicons, were detected, comparing amplicons DNA size with the ladder bands. Electrophoresis analysis for DAT1/SLC6A3 gene is showed in Figure 22.

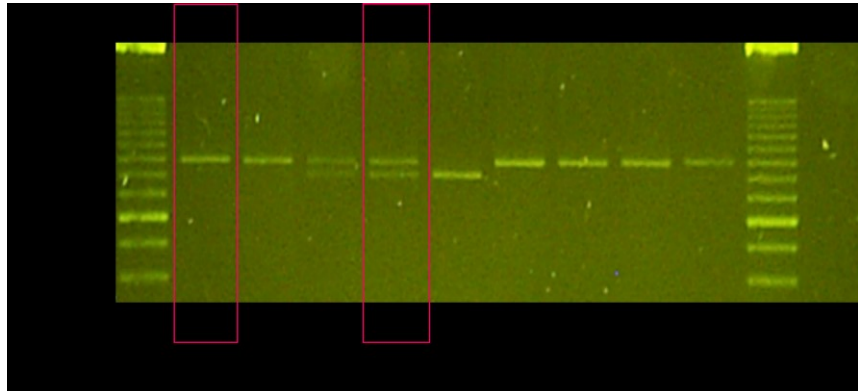


Figure 22. Agarose gel. 3'UTR 40 bp tandem repeat (repeated between 3–13 times) with 9 and 10-repeat allele being the most frequently found in the human.

In case of SNPs, the PCR products were subjected to restriction digestion, before electrophoresis analysis. A single nucleotide variation can lead to presence or absence of a restriction site, which are cleavage sequences recognized by restriction enzymes. In this condition, verified for rs4680 and rs1800497, COMT and ANKK1 genes respectively, restriction digestion reaction on PCR amplicons allowed to successful distinguished the allelic variants. Restriction sites can be used as markers for quick and easy identification of alleles in a specific position. When the restriction site was not located in the SNP region (as in the case of the others SNPs), mutagenic primers were used to create the cleavage sequence artificially, as shown in Table 3.

Gene	Polymorphism	Primer Forward (5'-3')	Primer Reverse (5'-3')	Reference
CNR1	rs1049353	GAAAGCTGCATCAAGAGCCC (1)	TTTTCCTGTGCTGCCAGGG	Gadzicki et al., 1999
FAAH	rs324420	ATGTTGCTGGTTACCCCTCTCC (2)	TCACAGGGACGCCATAGAGCTG	Morita et al., 2005
COMT	rs4680	TCGTGGACGCCGTGATTCAGG	AGGTCTGACAACGGGTCAGGC	Hong et al., 2003
DRD2	rs6277	a) ACCACGGTCTCCACAGCACTCT (3) b) ACCATGGTCTCCACAGCACTCT'	ATGGCGAGCATCTGAGTGGCT	Hirvonen et al., 2009
ANKK1	rs1800497	CCGTCGACGGCTGGCCAAGTTGTCTA	CCGTCGACCCTTCCTGAGTGTCATCA	Grandy et al., 1993
DAT1	VNTR 3'UTR	TGTGGTGTAGGGAACGGCCG AG	CTTCCTGGAGGTCACGGCTCAAGG	Santtila et al., 2010
DRD4	VNTR exon 3	AGGTGGCACGTCGCGCCAAGCTGCA	TCTGCGGTGGAGTCTGGGGTGGGAG	Mitsuyasu et al., 2001

Table 3. List of the primers used for PCR amplification. Highlightened in red the mutagenic nucleotides added in order to create digestion restriction sites.

For the rs1049353 ([1]) SNP in CNR1 gene, a C mismatch creates an artificial restriction site for MspI, site that is canceled from the substitution G →A (Gadzicki et al., 1999). In the FAAH gene, a T mismatch ([2]) allows the insertion of a thymine instead of adenine and remove a restriction site for EcoO109I, rs324420 SNP (Morita et al., 2005). In the case of rs6277 of DRD2 gene, the T mismatch ([3]), located in the 3' primer sequence, generates a restriction site for Taq$^{\alpha}$I enzyme in the PCR product when for C allele carrier, restriction site is absent in allele T carrier. In addition, rs6277 amplification needs a double forward primer (FWa e FWb), because of the presence of a SNP in the primer pairing sequence (Hirvonen et al., 2009).
As example SNP electrophoresis analysis for the SNP rs1800497, ANKK1 gene, is reported in Figure 23: the amplified PCR fragments were digested with Taq^{a}I restriction enzyme and the digested fragments were visualized by agarose gel electrophoresis. As showed in Figure 23 after PCR products were loaded on agarose gel was possible to recognize CC(A2/A2) carriers, indicated by two fragments of 184 bp and 134 bp, TT (A1/A1) carriers, who showed the uncleaved 318 bp fragment and hetherozygous CT (A1/A2) revealed by all the three fragments 318 bp, 184 bp and 134 bp.

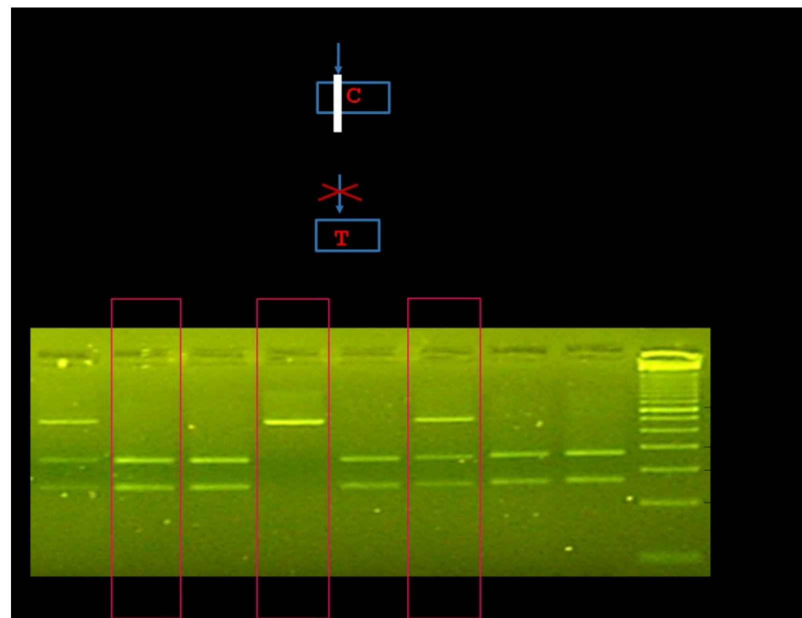


Figure 23. Electrophoresis analysis for the SNP rs1800497, ANKK1 gene.

All the PCR products were digested with proper restriction endonucleases for each SNP analyzed and all genotypes were readily distinguishable from each other.

3.1.4 Genotyping method 2

American population

The genotyping procedure in the American population was carried out in three main steps: biological samples collection, DNA extraction and TaqMan Genotyping Assay.

3.1.4.1 Biological sample collection

The samples for the American study were previously collected for the project #12-DA-N472, NIDA, IRP [Health Outcomes by Neighborhood (HON) – Baltimore]. To achieve this research, approximately 5 ml aliquots of whole blood were provided for each subject.

3.1.4.2 Genomic DNA extraction

Blood samples were thawed at room temperature in a biosafety cabinet and immediately subjected to the DNA purification protocol from whole blood using the QIAamp DNA Blood Midi/Maxi Kit (Spin Protocol). The blood samples were lysed with QIAGEN protease and Buffer AL. After lysis, the lysate is loaded onto the QIAamp spin column, DNA bound to the QIAamp membrane and impurities were washed away in two centrifugation steps. Finally, genomic DNA was eluted, reloading the elute twice to obtain maximum concentration (Figure 24).

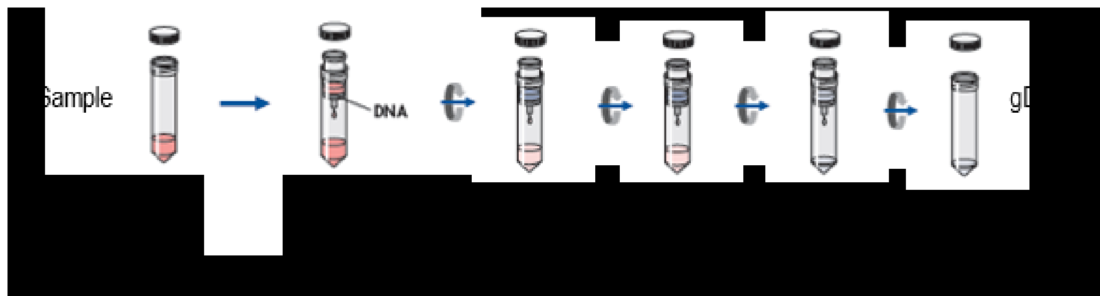


Figure 24. The QIAamp DNA Blood Midi and Maxi Spin Procedures

After DNA extraction, all the samples have been quantified with NanoDrop 2000c Spectrophotometer (Thermo Scientific) and divided in 4 aliquots: 20 ng gDNA reserved for epigenetic analysis, 10 ng gDNA reserved for genetic analysis and the remaining volume was kept separately.

3.1.4.3 TaqMan® genotyping Assay

TaqMan®genotyping assays (Thermo Fisher) genotype single nucleotide polymorphisms (SNPs) in purified genomic DNA samples. Each assay contains two primers for amplifying the sequence of interest and two TaqMan®MGB probes for detecting alleles and the presence or absence of a SNP is determined based on the change in fluorescence of the dyes associated with the probes.
The reaction mixture was prepared, consisting of TaqMan Genotyping Master Mix, forward and reverse primers and two TaqMan MGB Probes (Table 4) and was added to gDNA.
Each probe is labeled with a different fluorophore (VIC or 6FAM), attached covalently to the 5' end of the two probes. During the PCR reaction (Figure 25), when probes hybridize specifically to complementary DNA fragments, because of the 5´→ 3’ nuclease activity of the AmpliTaqGold DNA Polymerase (ultra-pure to minimize nonspecific, false positive DNA products due to bacterial DNA contamination during PCR), contained in the master mix, they are destroyed and the fluorescence of corresponding fluorophore is liberated. Near the 3' end, there is a non-fluorescent quencher (NFQ) that prevents liberation of the reporter fluorescence if the probe is not degraded: the proximity of the probe with a quencher dye suppresses the reporter fluorescence. A minor groove binder (MGB) stabilize the double-stranded structure formed between the target and the probe.

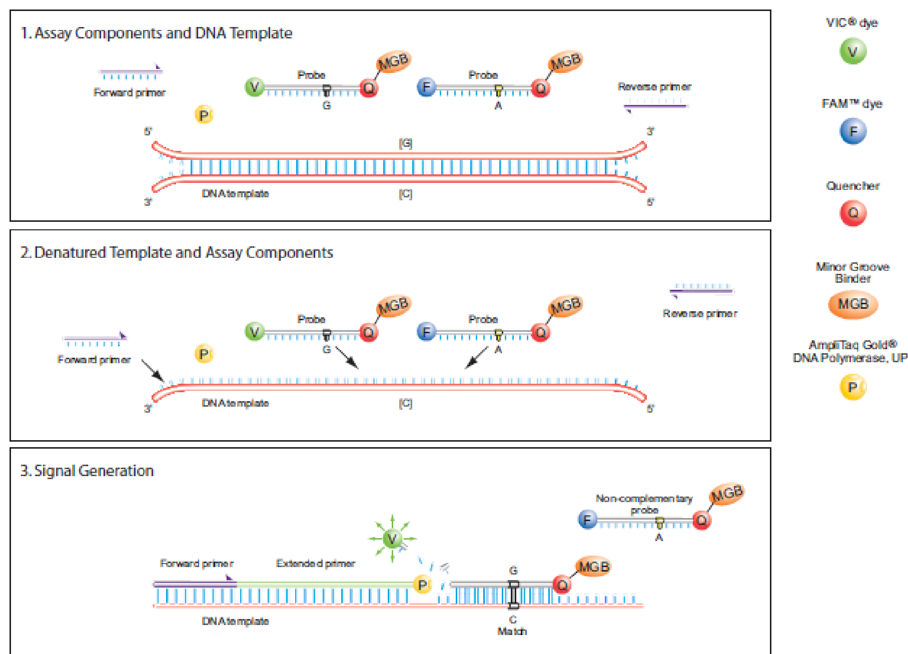


Figure 25. Basics of TaqMan Genotyping Assay

The increase in fluorescence occurs only if the amplified target sequence is complementary to the probe. Thus, the fluorescence signal generated by PCR amplification indicates which alleles are in the sample, as shown in Table 4.

Fluorescence Increase	Indication
VIC dye fluorescence only	Homozygosity for allele 1
6FAM dye fluorescence only	Homozygosity for allele 2
Fluorescence signals for both dyes	Heterozygosity for allele 1-allele 2

Table 4. Fluorescence signal correlations

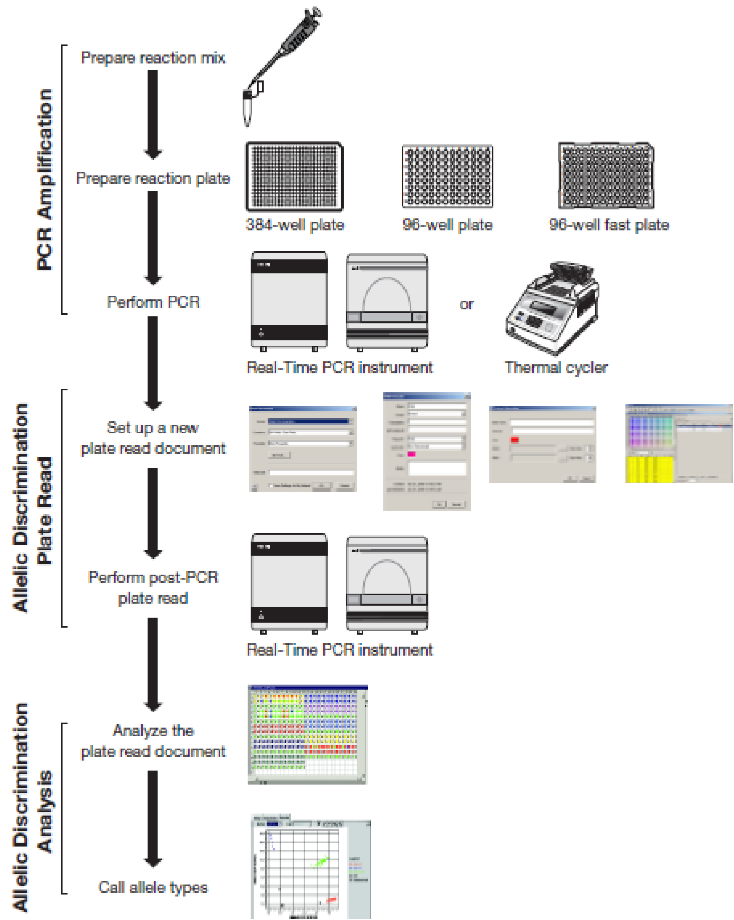


Figure 26. Overview of genotyping workflow.

The SDS software results at the end of the process in a plot allowing the allelic discrimination. Each well-reaction plate is represented as an individual point on the plot (all the plots from the experiments conducted in this study are reported in the Results section (pages 61-65, Figures 36-43).
SDS software allows to automatically generate the baseline values (the initial cycles of PCR where little change in fluorescence is measured) for each well and a level of ΔR_n, used for C_T determination in real-time assays.

R_n is the normalized reporter, the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye (the reporter dye is the dye attached to the 5′ end of a TaqMan® probe: it provides a fluorescent signal that indicates specific amplification). And C_T is the fractional cycle number at which the fluorescence passes the threshold (Figure 27). ΔR_n is thus the magnitude of the signal generated by the specified set of PCR conditions. (ΔR_n = Rn − baseline).

The level of C_T is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the amplification plot defines the C_T.

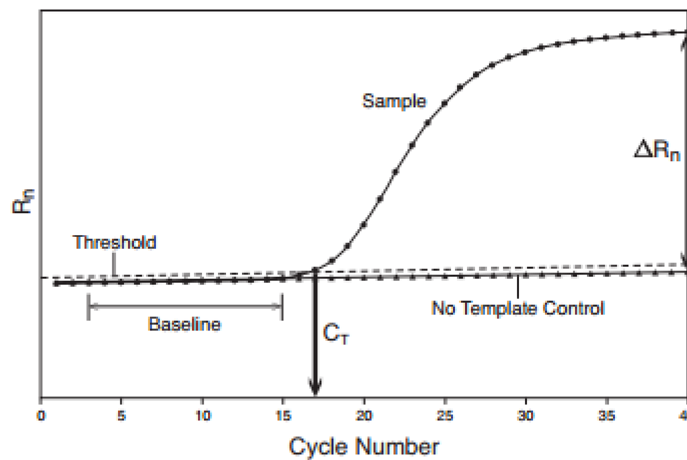


Figure 27. A representative amplification plot.

The polymorphisms of CNR1 and CNR2 genes, listed in Table 5, were genotyped using TaqMan-based analysis. Genotyping was performed in a volume of 5 µl containing 10 ng of genomic DNA (2 µl of 5 ng/µl).

Gene	Polymorphism	Chr position	Assay id	[VIC/FAM]
ANKK1	rs1800498	Chr.11: 113420866	C___2601166_10	[A/G]
CNR1	rs1049353	Chr.6: 88853635	C___1652590_10	[C/T]
	rs2180619	Chr.6: 88877952	C__15841551_10	[A/G]
	rs806379	Chr.6: 88861267	C___1652584_10	[A/T]
	rs6454674	Chr.6: 88872930	C__11418433_10	[G/T]
	rs12720071	Chr.6: 88851181	C__30749291_10	[C/T]
	rs2023239	Chr.6: 88860482	C__11600616_10	[C/T]
CNR2	rs2501431	Chr.1: 24201643	C____515482_10	[A/G]

Table 5. Polymorphisms analysed with TaqMan Genotyping Assay. Assay ID and [VIC/FAM association] is reported.

The 2 µl volume of gDNA (5 ng/µl) for each sample were previously prepared in a 384-well plate and subsequently added 3-µL PCR Reaction mix (Table 6) in each well. Amplification was performed on a commercially available system (ViiA7, Life Technologies), starting with 30 sec at 60 °C, followed by 10 min at 95°C. 40 cycles of 15 s at 95 °C were than performed and 1 min at 60 °C. Genotypes were scored using the algorithm and software supplied by the manufacturer.

Component	Volume	Final concentration
TaqMan Genotyping Master Mix (2×)	2.5 µl	0,4 - 4 ng/µl
TaqMan genotyping assay mix (20×)*	0.25 µl	1X
DNase/RNase-free water	0.25 µl	1.5 mM
Total	3 µl	0.2 mM each

Table 6. PCR reaction mix volume. Reaction mix component needed for each assay. 40× Assay Mix was diluted 1:2 with 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

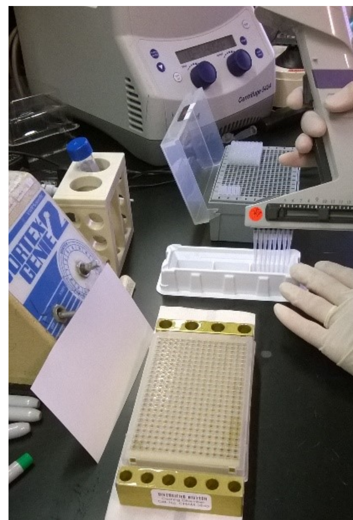


Figure 28. Performing Taqman Genotyping Assay in the 384 well plate.

3.2 ENVIRONMENTAL STUDY

Caucasian population

To find out if environmental factors could affect the risk of cannabis use disorders, in Caucasian population study, each subject was submitted to three psychometric tests:

- ACEs (Adverse Children Experience Scale)
  This questionnaire allows to evaluate adverse childhood experiences prior to18th birthday, as physical, sexual and emotional abuse, physical and emotional neglect. It also includes, having a parent who is mentally ill, an alcoholic or substance abuser, in jail, or a victim of domestic violence, as well as the absence of a parent through divorce, death or abandonment (Centers for Disease Control and Prevention, 2010).

- CECA-q (Child Experience of Care and Abuse Questionnaire)
  The CECA interview is a semi-structured, retrospective interview used to determine many adverse experiences encompassing neglect, antipathy, physical abuse, and sexual abuse among others (Bifulco et al., 1994)

- PBI (Parental Bonding Instrument)
  It is a widely-used measure of parenting, how a person's parents behaved towards him or her in childhood. It is usually used to measure care, overprotection and control (Parker et al., 1978).

American population

In this study, demographic data have been considered as environmental factors that could influence cannabis use disorders; the environmental variables were gender, marital status, level of education, BMI (Table 7).

	Environmental variables	Details
Caucasian population	**gender**	male vs female
	ACEs	
	Emotional abuse	
	Physical abuse	
	Household dysfunction	
	Emotional neglect	yes/no
	Physical neglect	
	Parental separation	
	Parental mental illness	
	Sexual abuse	
	CECAq	
	Antipathy mother	
	Antipathy father	
	Neglect mother	
	Neglect father	yes/no
	Phys. abuse mother	
	Phys. abuse father	
	Sexual ab. Screen	
	Sexual ab. Severity	
	PBI	
	neglectful parenting	
	affectionless control	score
	affectionate constrain	
	optimal parenting	
American population	**gender**	male vs female
	marital status	married vs not married
	level of education	1= some high school/GED 2= H.S. diploma 3= some college 4= college graduate/Masters/Ph.D
	BMI	<25/≥25

Table 7. Environmental variables details for Caucasian and American studies.

3.3 EPIGENETIC STUDY

American population

3.3.1 Methylated DNA Immunoprecipitation (MeDIP)-qPCR

Different strategies are available to identify chromosomal sites of DNA methylation: use of methylation-sensitive restriction enzymes, that unfortunately require high-molecular-weight DNA and are limited by the restriction site of the specific enzyme (e.g. Hpa II, Msp I) (Fazzari et al., 2004); bisulfite methods (based on the conversion of unmethylated cytosine), following by sequencing, represent a sensitive alternative, but it is laborious and restricted to few samples analyzed at a time (Rakyan et al., 2004). For these reasons for epigenetic analysis methylated DNA immunoprecipitation (MeDIP) technique has been used (Figure 29), followed by quantitative PCR, developed by Weber and coworkers (2005). MeDIP-qPCR is one of the most powerful approaches described to interrogate for regional DNA methylation changes based on the chromatin immunoprecipitation of methylated fragments (Weber et al., 2005). The procedure includes 4 steps: isolation of genomic DNA (gDNA), sonication of gDNA, immunoprecipitation of methylated genomic fragments, using an antibody specific for methylated cytosines and quantitative Real Time PCR.

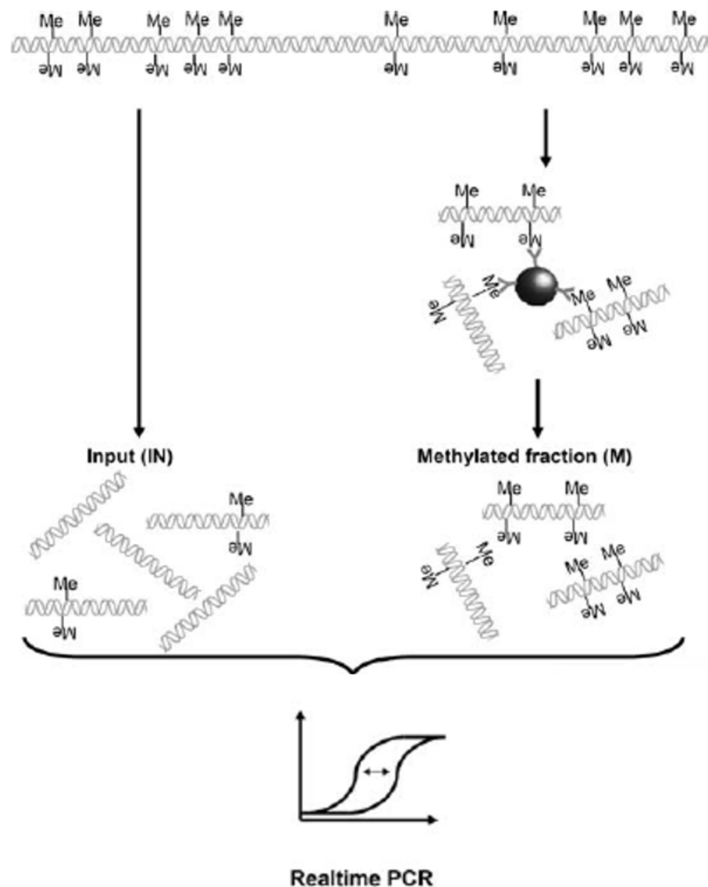


Figure 29. MeDIP-qPCR resumed steps (Weber et al., 2005).

3.3.1.1 Isolation of genomic DNA

To quantify DNA methylation in specific sites of genes involved in the neurobiology of cannabis use disorder 20 ng aliquots of the gDNA, previously extracted for TaqMan genotyping assay in the American population, have been used.

3.3.1.2 Sonication of gDNA

Genomic DNA has been randomly sheared and subjected to denaturation by sonication to generate fragments between 300 bp and 1000 bp. Diagenode Biorruptor has been used. A checking session has been run to decide the number of cycles to reach ~200–600 bp DNA fragments size. Six cycles session gave the most homogenously result (Figure 30 reports the sheared DNA set). Systematic checking of the sheared DNA size on agarose gel to ensure equal sonication has been conducted after shearing session. For all the sample the shearing process included 6 cycles, 30 s "ON", 30 s "OFF".

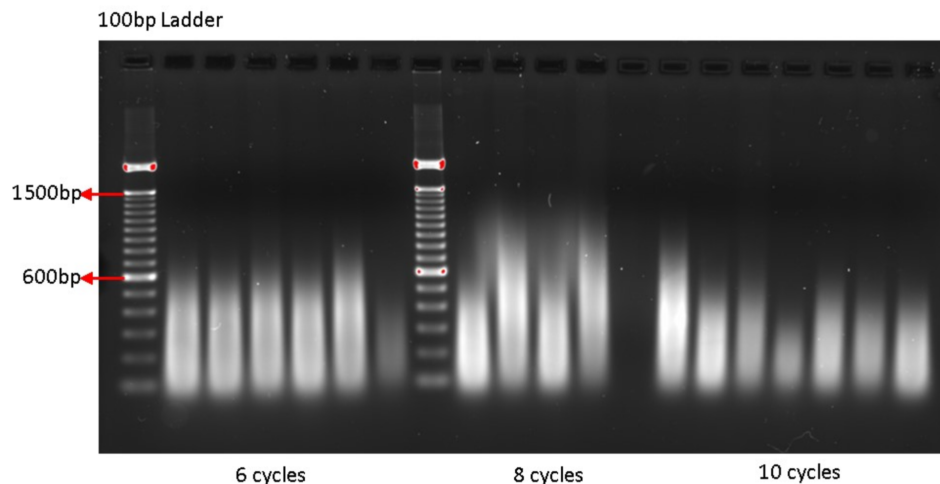


Figure 30. Checking of the sheared DNA size, after sonication, on TAE 2% agarose gel, post-stained with 1:25 TAE EtrBr.

3.3.1.3 Immunoprecipitation of methylated genomic fragments

58 sheared samples among control subjects and 38 marijuana users have been selected for immunoprecipitation and DNA methylation analysis.

Immunoprecipitation

The sonicated and denatured gDNA (5µg) was immunoprecipitated overnight at 4°C using 5 µl of a monoclonal antibody against 5mC (anti-5-methylcytosine, 5-mC, mouse monoclonal antibody [33D3], Millipore), 50 µl IP Buffer (10 mM sodium phosphate (pH 7.0), 140 mM NaCl, 0.05% Triton X-100), in a final volume of 500 µl TE.

A portion of the sonicated DNA was left untreated to serve as input control. Input control is a fraction of the same gDNA, but not immunoprecipitated with the antibody, collected prior to immunoprecipitation,

which thus includes methylated and not methylated DNA. These sheared "input" DNA samples are essential for later comparison with immunoprecipitated DNA. The input was prepared with the same concentration of the samples: 20 ng in 3 µl (6.67 ng/ µl).

Magnetic Beads incubation

The beads were previously washed with PBS 0.1% BSA and re-suspended on 40 µL of 1X IP buffer. Dynabeads (Life Technologies) were subsequently incubated overnight at 4°C.

DNA isolation

Dynabeads were washed three times with 700 µl of IP buffer and treated with proteinase K for 3 h at 50°C and recovered the methylated DNA by phenol-chloroform extraction followed by ethanol precipitation. DNA samples have been finally quantified with Nanodrop, 2000c Spectrophotometer (Thermo Scientific).

3.3.1.4 Real Time qPCR

Primer Design

Enrichment DNA methylation was determined by quantitative real-time PCR using specific ChIP primers designed to amplify proximal or distal sequences from the transcription start site (TSS). Primers were designed using BLAST Pick up Primer Tool, following as primer criteria selection: PCR product size between 125-300 bp, primer size Min (20)-Opt (23)-Max (25), GC content 40-70%, including all the possible CpG regions. The CpG regions position was investigated using UCSC genome browser (on Human Dec. 2013 (GRCh38/hg38) Assembly; https://genome.ucsc.edu/cgi-bin/hgGateway). Details on primer sites annealing are reported in the Results session (pages 70-76).
The specific primer sequences used in this study are listed in Table 8.

Description	Forward Primer	Reverse Primer
Chip Rn_ANKK1 -0.25 TSS	TGGACTTCTTCCAGCTT	GGGAAATCCCAGACATGA
Chip Rn_DRD2 -0.4 kb TSS	CAGCAATAGAACTGACTACA	GCTCGCCAGTCTTCTCCTTTGA
Chip Rn_DRD2 +0.9 kb TSS CpG	CAACTCTGTGTTGGTGC	GTGTCAACCCAAGAGAAG
Chip Rn_DRD2 +66.7 kb exon8	CTAAGAGGCTGCTGAAAACCATC	GACACAGGGGTCTTGCTATACTT
Chip Rn_CNR1 +22.31 kb SNP region (rs1049353)	CAATCTTGACCGTGCTCTTGATG	GAGCATGTTTCCCTCTTGTGAAG
Chip Rn_NCAM1 +0.4 kb TSS	GGCGTAGGGTAGAAGTGTGAAAA	CCGAACATCAAGGAGGTAAGAGA
Chip Rn_NCAM1 +3 kb TSS CpG	GATATTTGGTGGGCCTTTTGGG	TTCCAGGGAACACTGGTTAGAAT

Table 8. ChIP primers designed for Real Time PCR on immunoprecipitated methylated gDNA.

Real Time qPCR

We carried out qPCR reactions with 20 ng of input DNA and immunoprecipitated methylated DNA. For qPCR reactions, we used the iQ SYBR Green PCR master mix (Bio-Rad) and Roche thermal cycler (Roche Diagnostics).

During PCR, SYBR Green intercalates into the dsDNA helix and the increase in SYBR Green fluorescence is directly proportional to the amount of dsDNA generated. In solution, the unbound dye exhibits very little fluorescence; however, fluorescence (measured at 530 nm) is greatly enhanced (100-fold) upon binding to DNA due to conformational changes.

Component	Volume	Final concentration
SYBR Green PCR master mix (Bio-Rad)	10 µl	
Primer	0.5 µl	0.5µM
Primer	0.5 µl	0.5µM
H_2O	6 µl	
Total	17 µl	

Table 9. Real Time PCR reaction mix.

To determine whether only your desired PCR product has been amplified (and to exclude double-stranded PCR artifact, which can contribute to signal intensity, non-specific products and primer-dimers), we performed a Melting Curve analysis after PCR (Figure 31). PCR product characterization by Melting Curve analysis is based on the fact that each particular double-stranded DNA molecule has its characteristic melting temperature T_m, at which 50% of the DNA is double-stranded and 50% is melted, single-stranded. During the melting curve run, the instrument continuously monitors fluorescence, the mixture was slowly heated up to 95°C, which causes melting of dsDNA and a corresponding sharp decrease of SYBR Green fluorescence when PCR product reach their T_m: this is visualized in a plot (fluorescence vs T). To better distinguish samples and their non-specific products the first negative derivative of the melting curve was plotted, for each reaction, and T_m was displayed as a peak; additional melting peaks result from primer-dimers or other non-specific products.

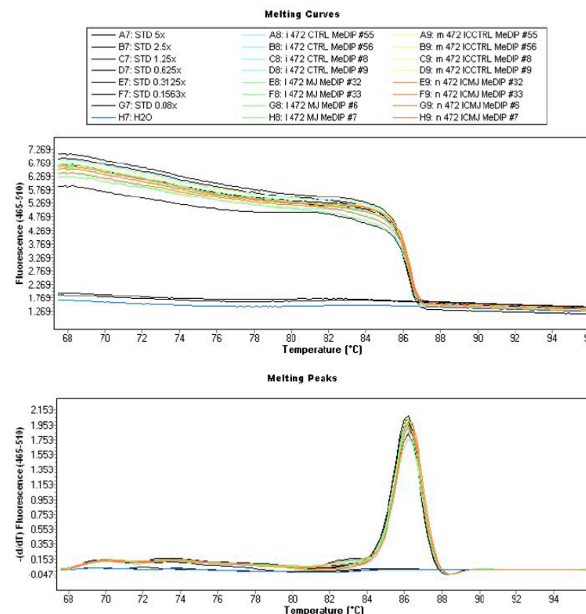


Figure 31. Melt Curve and Melting Peak for NCAM1 +3 CpG (Melt Curve Genotyping).

MeDIP data analysis

Real Time PCR reactions were performed using LightCycler® 480 Instrument (Roche). The data obtained from the instrument have been subjected to Absolute Quantification analyses, based on Second Derivative Maximum method (Figure 32) that allows to quantify a single target sequence and expresses the result as an absolute value.

The reaction profile contains three phases: an initial background phase, that lasts until the fluorescence signal from the PCR product is greater than the background fluorescence, an exponential (log-linear) phase, where sufficient product has accumulated to be detected above background, and a plateau phase: the reaction efficiency falls and the reaction enters the plateau.

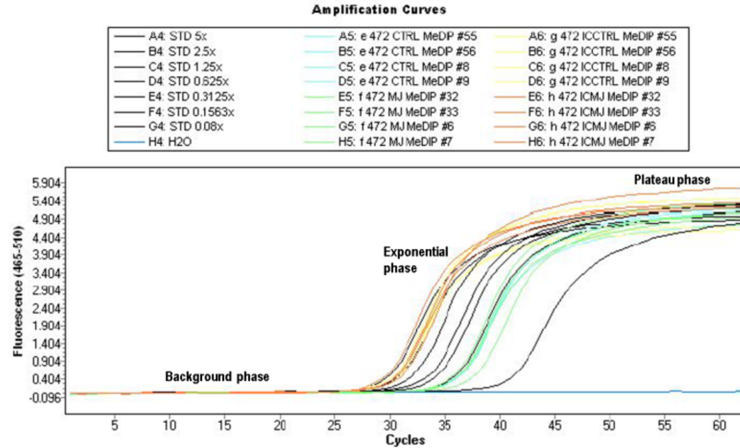


Figure 32. Abs Quant/2nd Derivative Max for NCAM1 +0.4 (Abs Quant/2nd Derivative Max).

The point at which the fluorescence of a sample rises above the background fluorescence, where the sample's fluorescence curve turns sharply upward, is called the "crossing point (Cp)" of the sample. This turning point corresponds to the maximum of the second derivative of the amplification curve. Thus, this method is called "Second Derivative Maximum method" and requires little user input.

To determine the concentration of unknown samples a standard curve was used (Figure 33): the concentrations of standard samples are plotted against the Cps of the samples. The X axis represents the log of the initial target concentration, and the Y axis represents Cp in cycles. The software performs the calculation automatically and can thus determine the initial concentration of target DNA in the sample by determining where an unknown sample's crossing point falls on the standard curve.

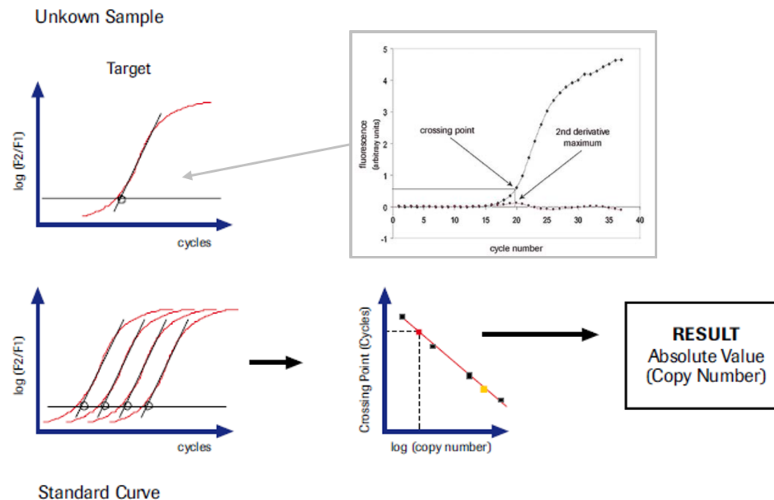


Figure 33. The role of standard curve.

Standards preparation

Seral dilutions of an external standard with predefined known concentration were used to create the standard curve (Figure 34). The standard dilutions were amplified in the same LightCycler® 480 Instrument run. The crossing points of standards and unknown samples were in this way used to determine the concentration of target DNA. Specifically, high and low standards have been prepared from a mixture of all the input controls (20ng/µl), derived mixing input controls from both analyzed groups (marijuana users and control subjects). Serial dilutions were executed to obtain high and low standard.

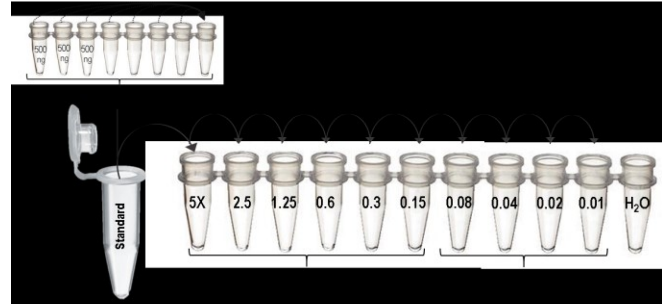


Figure 34. Serial dilutions for standards preparation.

The *Slope* of the standard curve describes the kinetics of the PCR amplification: how quickly the amount of target DNA can be expected to increase with the amplification cycles. It is referred to the *Efficiency* of the amplification reaction: a perfect amplification reaction has the efficiency of 2 (Figure 35) and the *Error* value, that is a measure of the accuracy of the quantification result based on the standard curve, is considered an acceptable value when is < 0.2.

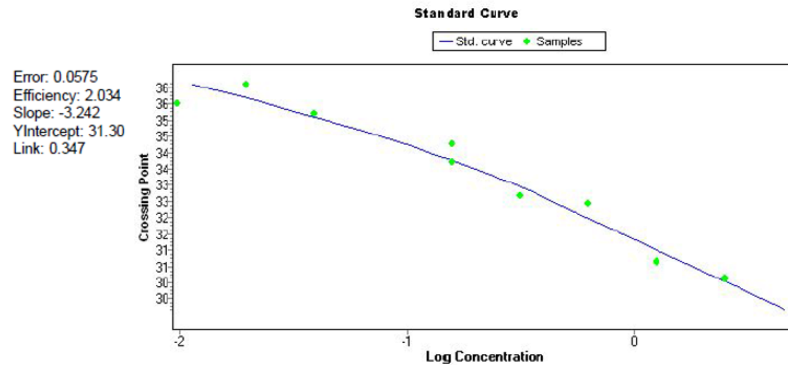


Figure 35. The quantification experiment (Abs Quant/2nd Derivative Max for NCAM1 +0.4).

To evaluate the relative enrichment of target sequences after MeDIP, we calculated the ratios of the signals in the immunoprecipitated DNA versus input DNA.

For each reaction, average, standard deviation and standard error of the ratio samples/input controls have been calculated. In addition, a table has been created to calculate the range of significance and which outliers should be removed from the sample/IC data. Fold change has been calculated as the concentration of each sample divided by the average of the control group.

3.4 STATISTICAL ANALYSIS

Statistical analysis of genetic data

The associations between marijuana use and allele frequencies and genotype distributions of all the polymorphisms were determined using Fisher's exact test.

Haplotype frequencies, haplotype odds ratio (OR) and 95% confidence interval (95% CI), and pairwise linkage disequilibrium (LD) were estimated. Haplotype frequencies were determined by using PLINK (1.07, Author: Shaun Purcell, URL: http://pngu.mgh.harvard.edu/purcell/plink/).

The SNPs involved in haplotypes analysis were the six SNPs of CNR1 on chromosome 6 (rs1049353|rs806379|rs6454674|rs2023239|rs12720071|rs2180619) for American population and the two SNPs of chromosome 11 (rs1800497|rs6277, ANKK1 and DRD2 respectively) for Caucasian population.

The analysis was conducted by the sliding window method using 2-5 or 6 marker windows (shifting one SNP at a time) and by the haplotype based case/control association. The haplotype based case/control association is basically the sliding window test using all the six SNPs.

The sliding windows methods form the SNP haplotypes across the entire dataset, respecting chromosome boundaries. The haplotype based case/control association is used for population-based sample of unrelated individuals.

Statistical analysis of environmental data

Logistic regression was used to assess the association between psychometric test (for Caucasian population) or demographic variables (for American population) and marijuana use.

For the Caucasian subset, three logistic regression models were run: the first two models evaluate the effects of psychometric variables on marijuana use. The variables that were statistically significant ($p<0.05$) correlates of marijuana use in the first two models were included in a third logistic regression model, along with genotype variables.

For the American subset, four independent variables were evaluated (gender, marital status, BMI, educational level) (Table 10). All the four variables were entered into the model in this initial step: gender (male vs. female), education (divided in four categories: 1.Some High School/GED, 2. High School Diploma, 3.Some College, 4. College Graduate, Masters, Ph.D), BMI (≥ 25 vs. <25), and marital status (married vs. not married). A backwards elimination procedure was used, in which one non-significant variable was removed at a time until only statistically significant ($p<0.05$) predictors remained. BMI was removed in step 2 and marital status in step 3. The last step (step 3) shows the final model with only two significant variables remaining: education and gender. Gender was used as a categorical variable and education was used as a numeric variable.

A second logistic regression model aimed to determine the demographic and genetic correlates of marijuana use in the American population. This model included all four demographic variables (gender, marital status, BMI, and education) as well as eight SNP allele types as independent variables. The database was set up so that each SNP (e.g. rs1800497 ANKK1) was a separate variable, and there were two rows per participant containing the two alleles for that SNP (e.g. genotype AG would have A in the first row and G in the second row). Generalized Estimating Equations (GEE) were used to account for within-subject correlations; an exchangeable correlation matrix was specified. The GEE procedures were run using SAS version 9.4 (SAS Institute, Cary NC); all other statistical analyses were conducted IBM SPSS (version 24).

For all the statistical analyses, results were considered statistically significant if the p value was less than 0.05.

		MJ use	
		No	Yes
Gender	Male	38	30
	Female	58	10
Marital Status	Not married	84	38
	Married	12	2
BMI	<25	36	18
	≥25	55	19
Education	1= some high school/GED	12	12
	2= H.S. diploma	24	10
	3= some college	33	16
	4= college graduate/Masters/Ph.D	27	2

Table 10. Custom Table (SPSS) of the 4 demographic variables (gender, marital status, BMI, educational level) evaluated on the dependent variable, marijuana use (MJ use) in the American subset.

Statistical analysis on epigenetic data

MeDIP-qPCR statistical analysis were performed using STATVIEW 5.0. All the quantitative data are presented as mean_SEM. For data comparing controls (CTRL) and marijuana users (MJ) groups, unpaired Student t test was used (StatView version 5.01, St. Louis, Missouri).

4. Results

4.1 CANDIDATE GENE ASSOCIATION STUDY IN CAUCASIAN POPULATION

Genetics analysis were conducted on 93 control subjects and 92 marijuana users. Biological samples (buccal cells or whole blood) collected from these participants have been genotyped for the following genetic polymorphisms: rs1049353 (CNR1 gene), rs324420 (FAAH gene), rs4680 (COMT gene), rs6277 (DRD2 gene), rs1800497 (ANKK1 gene), VNTR 3'UTR (DAT1/SLC6A3 gene), VNTR exon 3 (DRD4 gene). In the section Methods, Table 1 reports the list of the polymorphisms analysed, DNA sequence variations and functional consequences.

Tables 11 through 17 report results from genotyping for each analysed polymorphism.

SNP rs1049353 CNR1 gene

Genotypes relating to G1359A SNP of CNR1 gene show low frequency of homozygous A/A genotype in both groups. Homozygous G/G are more frequent in the control group (59.14%), than marijuana users (52.17%). Heterozygous G/A carriers are instead more frequent among marijuana users (47.83%) than controls (36.56%). G allele is slightly more represented among controls and A allele in the marijuana users group. Statistical analysis showed no significant differences at allelic level (*Fisher's exact test* (A) =0.80), but differences result in the genotypes analysis (*Fisher's exact test* (G) = 0.051) (Table 11).

	CTRL subjects (n=93)	**MJ users** (n=92)
Genotypes		
Homozygous G/G	59.14%	52.17%
Homozygous A/A	4.30%	0.00%
Heterozygous G/A	36.56%	47.83%
Alleles		
G allele	77.42%	76.09%
A allele	22.58%	23.91%

Fisher's exact test (G) = 0.051
Fisher's exact test (A) = 0.80

Table 11. Genotypes distribution and allelic frequencies relating to the SNP rs1049353, CNR1 gene.

SNP rs324420, FAAH gene

Homozygous C/C genotype is the genotype with higher frequency for the SNP rs324420 of FAAH gene in both groups, with a little higher frequency in the marijuana users group (68.48%), compared to controls (62.37%). Homozygous A/A genotype is more frequent among controls (6.45%), than cannabis users (3.26%); heterozygous AC genotype is almost homogeneously distributed (31.18% control group and 28.26% cannabis users). No significant variations were highlighted with statistical analysis between controls and marijuana users (*Fisher's exact test* (G) = 0.52 e (A) = 0.29) (Table 12).

	CTRL subjects (n=93)	**MJ users** (n=92)
Genotypes		
Homozygous C/C	62.37%	68.48%
Homozygous A/A	6.45%	3.26%
Heterozygous A/C	31.18%	28.26%
Alleles		
C allele	77.96%	82.61%
A allele	22.04%	17.39%

Fisher's exact test (G) = 0.52
Fisher's exact test (A) = 0.29

Table 12. Genotypes distribution and allelic frequencies relating to the SNP rs324420, FAAH gene.

SNP rs4680, COMT gene

SNP rs4680 genotyping did not showed relevant differences between marijuana users and controls. Consistently, statistical analysis does not highlight significant differences (*Fisher's exact test* (G) = 0.97; (A) = 0.91) (Table 13).

	CTRL subjects (n=93)	MJ users (n=92)
Genotypes		
Homozygous G/G	33.33%	31.52%
Homozygous A/A	16.13%	16.30%
Heterozygous G/A	50.54%	52.17%
Alleles		
G allele	58.60%	57.61%
A allele	41.40%	42.39%

Fisher's exact test (G) = 0.97
Fisher's exact test (A) = 0.91

Table 13. Genotypes distribution and allelic frequencies relating to the SNP rs4680, COMT gene.

SNP rs6277, DRD2 gene

The most frequent genotype is heterozygous C/T, in both groups, controls (59.15%) and marijuana users (51%). Homozygous TT and CC are both slightly more frequent among marijuana users (33% and 16% respectively), compare to controls (29.03% and 11.83% respectively).
Alleles C and T are distributed homogenously. No significant differences result from statistical analysis (*Fisher's exact test* (G) = 0.51; (A) = 1) (Table 14).

	CTRL subjects (n=93)	MJ users (n=92)
Genotypes		
Homozygous C/C	11.83%	16%
Homozygous T/T	29.03%	33%
Heterozygous C/T	59.14%	51%
Alleles		
C allele	41.40%	41.85%
T allele	58.60%	58.15%

Fisher's exact test (G) = 0. 51
Fisher's exact test (A) = 1

Table 14. Genotypes distribution and allelic frequencies relating to the SNP rs6277, DRD2 gene.

SNP rs1800497, ANKK1 gene

SNP TaqIA genotyping results in a higher frequency of homozygous C/C (A2/A2) genotype among controls (76.34%), than cannabis users (57.61%); heterozygous T/C (A1/A2) is instead more frequent in the marijuana users group (38.04%, than 21.51% of control subjects). Differences have been shown even at allelic level: C (A2) allele is most represented in the control group (87.10%) compared to cannabis users group (76.63%); T allele on the contrary is more frequent among marijuana users (23.37%, compared to 12.90% in the control group).

	CTRL subjects (n=93)	MJ users (n=92)
Genotypes		
Homozygous C/C	76.34%	57.61%
Homozygous T/T	2.15%	4.35%
Heterozygous T/C	21.51%	38.04%
Alleles		
C allele	87.10%	76.63%
T allele	12.90%	23.37%

Fisher's exact test (G) = 0.034
Fisher's exact test (A) = 0.032

Table 15. Genotypes distribution and allelic frequencies relating to the SNP rs1800497, ANKK1 gene.

Statistical analysis reveals significant differences for both alleles (*Fisher's exact test* (A) = 0.034) and genotypes (*Fisher's exact test* (G) = 0.032) (Table 15).

VNTR-40 bp 3'UTR, DAT1 gene

In case of the 40 bp repeat in the 3'UTR of DAT1 gene, homozygous subjects for 10 repeats allele (homozygous 10R/10R) and heterozygous 9R/10R are the higher represented groups in the entire analysed population (about 43%). Some of the subjects presented the allele with 11 repeats, but the statistical analysis, that does not show significant differences between controls and cannabis users (*Fisher's exact test* (G) = 0.81; (A) = 0.73), has been done only for 9 and 10 repeats alleles, because the number of observations for 11 repeats was too low (Table 16).

	CTRL subjects (n=93)	MJ users (n=92)
Genotypes		
Homozygous 9R/9R	10.75%	7.61%
Homozygous 10R/10R	43.01%	44.57%
Heterozygous 9R/10R	43.01%	43.48%
Alleles		
9R allele	65.57%	67.78%
10R allele	34.43%	32.22%

Fisher's exact test (G) = 0.81
Fisher's exact test (A) = 0.73

Table 16. Genotypes distribution and allelic frequencies relating to the VNTR-40 bp 3'UTR, DAT1 gene.

VNTR-48 bp exon3, DRD4 gene

The genotypes related to the 48 bp VNTR of DRD4 gene are characterized by larger variability, because of the higher number of alleles. At population level, the most frequent genotype is homozygous 4R/4R (51.61% for controls and 56.52% for marijuana users). The most evident differences have been showed in the case of heterozygous 4R/7R, more frequent among controls (17.20%), compared to cannabis users (11.96%). Since the high number of alleles, the number of observations does not allow the statistical analysis for the genotypes distribution. For alleles analysis, the number of observation have been divided in two groups: less than 7 repeats (short) : <7 (S) and more than 7 repeats (long) ≥7 (L). Cannabis groups shows a slight majority of R<7 alleles, and R≥7 alleles are higher in the control groups, but no significant differences have been revealed (*Fisher's exact test* (A) = 0.36) (Table 17).

	CTRL subjects (n=93)	MJ users (n=92)
Genotypes		
Homozygous 3R/3R	1.08%	1.09%
Homozygous 4R/4R	51.61%	56.52%
Homozygous 7R/7R	2.15%	3.26%
Homozygous 2R/2R	1.08%	4.35%
Heterozygous 2R/4R	15.05%	11.96%
Heterozygous 2R/7R	5.38%	5.43%
Heterozygous 2R/8R	1.08%	0.00%
Heterozygous 3R/4R	3.23%	1.09%
Heterozygous 3R/8R	1.08%	0.00%
Heterozygous 4R/5R	0.00%	2.17%
Heterozygous 4R/7R	17.20%	11.96%
Heterozygous 7R/8R	1.08%	0.00%
Heterozygous 2R/3R	0.00%	1.09%
Heterozygous 4R/6R	0.00%	1.09%
Alleles		
R<7 (S) alleles	84.41%	88.04%
R≥7 (L) alleles	15.59%	11.96%

Fisher's exact test (A) = 0.36

Table 17. Genotypes distribution and allelic frequencies relating to the VNTR-48 bp exon3, DRD4 gene.

PLINK analysis was conducted by the sliding windows method. The SNPs involved in the haplotypes analysis were the 2 SNPs of chromosome 11 (rs1800497|rs6277, ANKK1 and DRD2 genes respectively) for Caucasian population. No significant association in Caucasian haplotypes analysis were found (Table 18).

Sliding win.	**SNPS**	**Haplotype**	**Frequency in MJ**	**Frequency in CTRLs**	**CHISQ**	**Degree of freedom**	**P**
	rs324420	A	0,1739	0,2204	1,264	1	0,2609
	rs324420	C	0,8261	0,7796	1,264	1	0,2609
	rs1049353	A	0,2446	0,2204	0,302	1	0,5826
	rs1049353	G	0,7554	0,7796	0,302	1	0,5826
2 SNPs	rs1800497|rs6277	OMNIBUS	NA	NA	5,613	3	0,132
	rs1800497|rs6277	TC	0,1732	0,1214	1,978	1	0,1596
	rs1800497|rs6277	CC	0,2398	0,2979	1,589	1	0,2075
	rs1800497|rs6277	TT	0,04961	0,01838	2,754	1	0,09704
	rs1800497|rs6277	CT	0,5374	0,5623	0,232	1	0,63
	rs4680	A	0,4239	0,414	0,0375	1	0,8464
	rs4680	G	0,5761	0,586	0,0375	1	0,8464

Table 18. Application of the haplotype sliding window method.

4.2 ENVIRONMENTAL STUDY IN THE CAUCASIAN POPULATION

To find out if environmental factors could affect the risk of addiction, in Caucasian population study, each subject was submitted to three psychometric tests (ACES, CECA-q, PBI), previously described.
The variables analysed in each test are listed below:

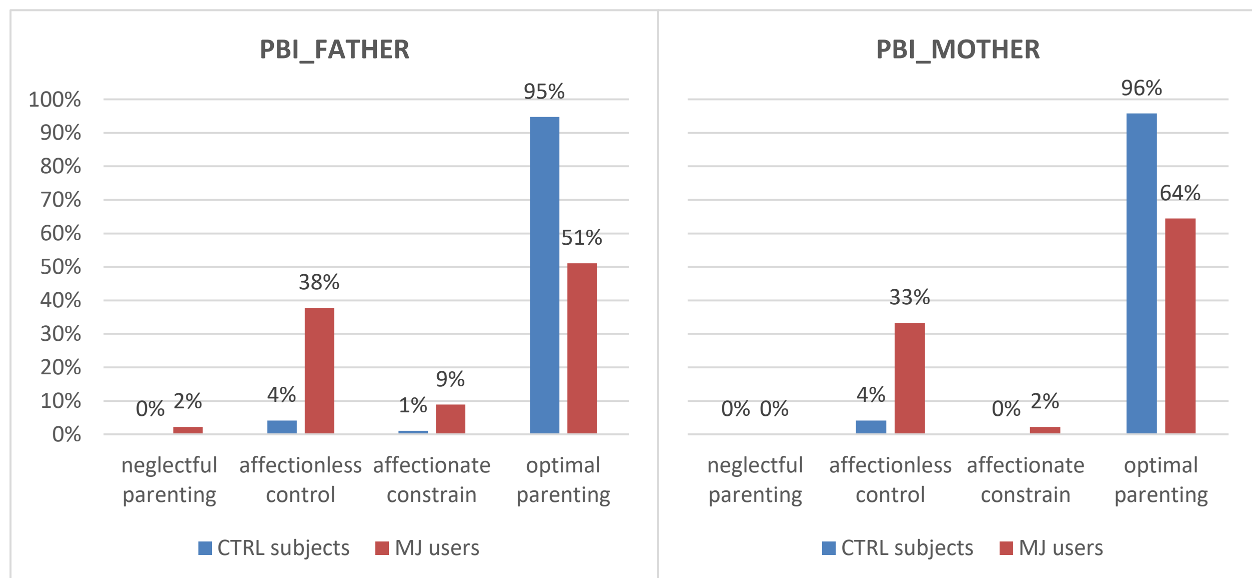
- PBI
  parental bonding, measured as neglectful parenting, affectionless control, affectionate constrain, optimal parenting

- ACEs
  emotional abuse, physical abuse, household dysfunction, emotional neglect, physical neglect, parental separation, parental mental illness, sexual abuse.

- CECA-q
  antipathy mother, antipathy father, neglect mother, neglect father, physical abuse mother, physical abuse father, sexual abuse screen, sexual abuse severity.

The following graphs highlight the differences between controls and marijuana users, concerning each variable measured.

Parental bonding instrument (PBI)



Adverse Childhood Experience (ACE) Questionnaire

The childhood experience of care and abuse questionnaire (CECA-Q)

Logistic regression models were estimated to highlight the effects of the single explanatory variables on addiction susceptibility. The first model evaluated the influence of gender and parenting on the dependent variable, namely marijuana use (Table 19). Bonding parenting was initially evaluated with PBI, to measure parental care, overprotection and control (types of bonding, measured with PBI, were precisely neglectful parenting, affectionless control, affectionate constrain, optimal parenting). Subjects reporting an optimal parenting were about 70% less likely to be (73% father; 63% mother) marijuana users than those who reported affectionless control or affectionate constrain. In addition, males present a six-time higher risk to develop cannabis use disorder compared to females.

Logistic regression — Number of obs = 140; Wald chi2(3) = 37.11; Prob > chi2 = 0.0000

Log pseudolikelihood = - 56.919186 — Pseudo R2 = 0.3469

Marijuana use	Odds Ratio	Robust Std. Err.	z	P>z	[95% Conf.	Interval]
gender	6,29015	3,23722	3,57	0	2,293989	17,24767
PBI father	0,27547	0,07988	-4,45	0	0,156047	0,486289
PBI mother	0,33167	0,12816	-2,86	0,004	0,155525	0,7073
_cons	14,59388	17,93741	2,18	0,029	1,312065	162,3254

Table 19. Logistic regression model 1. Explanatory variables: PBI father, PBI mother, gender; dependent variable: MJ use.

Logistic regression — Number of obs = 138; Wald chi2(15) = 42.70; Prob > chi2 = 0.0002

Log pseudolikelihood = -58.504081 — Pseudo R2 = 0.3032

	Marijuana use	Odds Ratio	Robust Std. Err.	z	P>z	[95% Conf.	Interval]
	gender	**3,208907**	1,562145	2,4	0,017	1,235888	8,331725
ACES	emotional abuse	0,5918614	0,6292269	-0,49	0,622	0,073668	4,755102
ACES	Physical abuse	2,555735	1,813362	1,32	0,186	0,636159	10,26753
ACES	Household dysfunction	0,3367997	0,5021392	-0,73	0,465	0,018126	6,258055
ACES	Emotional neglect	**12,15855**	11,75553	2,58	0,01	1,827659	80,88507
ACES	Physical neglect	**8,00004**	7,744079	2,15	0,032	1,199844	53,34079
ACES	Parental separation/divorce	0,8086108	0,9442513	-0,18	0,856	0,081989	7,974914
ACES	Household mental illness	0,7248716	0,4903002	-0,48	0,634	0,192536	2,729043
ACES	Sexual abuse	0,7102363	0,6225026	-0,39	0,696	0,127452	3,957835
CECAq	Antipathy mother	0,9099842	1,144551	-0,07	0,94	0,077342	10,70656
CECAq	Antipathy father	0,6141617	0,5729679	-0,52	0,601	0,098668	3,822887
CECAq	Neglect mother	0,7677557	0,9786799	-0,21	0,836	0,063121	9,338428
CECAq	Neglect father	2,946258	2,521796	1,26	0,207	0,550428	15,77035
CECAq	Physical abuse mother	1,729341	1,9471	0,49	0,627	0,190325	15,71324
CECAq	Physical abuse father	3,130648	4,278706	0,84	0,404	0,214924	45,60208
	_cons	0,0352549	0,029602	-3,98	0	0,0068	0,182783

Table 20. Logistic Regression model 2. Explanatory variables: gender, ACES and CECAq variables; dependent variable: MJ use.

To deepen different aspects of the parental bonding and adverse childhood experiences that could represent stressful life events, other psychometric tests were used: ACES and CECAq. Therefore, a second logistic regression model including gender, ACES and CECAq variables was run (Table 20). Three variables are statistically significant: *Emotional neglect, Physical neglect* and *Gender.* The coefficients of the first two (emotional neglect and physical neglect) are so high as to seem preconditions to addiction development: individuals reporting *emotional neglect* show a 12.2 times higher risk to develop marijuana abuse as well as those reporting *physical neglect* are 8 times more likely to develop cannabis addiction compared to subjects who do not have the perception of these psychological and physical damages. Finally, males are confirmed to present a statistically significant higher risk to develop cannabis use disorder compared to females. The significant variables coming out from this second model have been then included in a new model, where they were considered together with the genotypes (Table 21). None of the genetic variants have a significant association with marijuana use, whilst *emotional neglect* and *physical neglect* seem to be confirmed as preconditions to cannabis use disorders. Bonding parenting was not considered in this second model because of multicollinearity: PBI and some of the variables of CECAq and ACES are in fact closely associated.

Variables	Coefficients
Male (ref. Female)	4.632
Emotional neglect	24.950
Physical neglect	46.022
DAT1 (ref. 10R)	1.000
9R	1.565
9R/10R	1.211
others	3.429
COMT (ref. AA)	1.000
AG	3.788
GG	2.937
DRD2 (ref. CC)	1.000
CT	0.410
TT	0.852
ANKK1 (ref. CC)	1.000
CT	2.912
TT	0.348
FAAH (ref. AC)	1.000
CC	1.127
AA	0.590
CNR1 (ref. GA)	1.000
GG	0.898
DRD4 (ref No allele 4R)	1.000
Heterozygous 4R	2.743
Homozygous 4R	4.234
N	131
Addicted	41
Log-likelihood	-50.9

Table 21. Logistic Regression model 3. Explanatory variables: gender, physical/emotional neglect, genotypes; dependent variable: MJ use.

4.3 CANDIDATE GENE ASSOCIATION STUDY IN AMERICAN POPULATION

From February to November, 2016, I have worked in the Molecular Neuropsychiatry Research Branch (MNRB) of the National Institute on Drug Abuse (NIDA), Baltimore, MD, USA. There, I attempted to replicate the results of the identified genetic polymorphisms in a new cohort of patients that consisted of an American population of marijuana users and control subjects. Precisely, the SNPs analysed were the SNP rs1800497 of ANKK1 gene and others SNPs, belonging to cannabinoid CB1 and CB2 receptors genes (CNR1 and CNR2), including the previously analysed rs1049353 SNP of CNR1 gene. For this purpose, we extracted genomic DNA from 96 control subjects and 40 marijuana users and analysed the genetic polymorphisms by TaqMan Genotyping Assay technology (Thermo Fisher Scientific).
No significant differences were observed for the SNP rs1800497 of ANKK1 gene in the American population, comparing control subjects and marijuana users. Significant differences result instead genotyping rs1049353 SNP of CNR1 gene and the rs2501431 SNP of CNR2 gene; in both cases, differences have been reported concerning genotypes distributions. Tables 22 through 29 report results from genotyping for each analysed polymorphism and for each assay has been reported the allelic discrimination plot from the instrument (ViiA7, Life Technologies).

SNP rs1800497, ANKK1 gene

Taq1A allele (A allele, A1) is only a litte more frequent among marijuana users (23.3%), compared to controls (12.90%) in American population. No statistical differences were revealed for allelic frequency, nor genotypic distribution. Note: rs1800497 allele is reported in reverse orientation to the genome.

	CTRL subjects (n=96)	MJ users (n=39)
Genotypes		
Homozygous G/G	50,00%	51,28%
Homozygous A/A	9,38%	12,82%
Heterozygous G/A	40,63%	35,90%
Alleles		
Allele G (A2)	87.10%	76.63%
Allele A (A1)	12.90%	23.37%

Fisher's exact test (G) = 0.76
Fisher's exact test (A) = 0.88

Table 22. Genotypes distribution and allelic frequencies relating to the SNP rs1800497, ANKK1 gene.

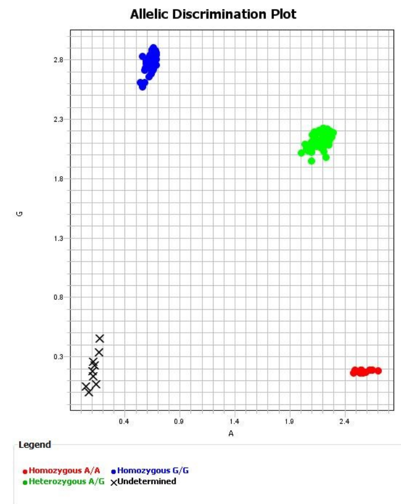


Figure 36. Allelic Discrimination Plot_ rs1800497

SNP rs1049353, CNR1 gene

As reported in the Caucasian population, low frequency of homozygous A/A genotype is observed in both groups. In the contrary, homozygous G/G carriers are more frequent among marijuana users (97.44%), than controls (78.13%) in this population and heterozygous G/A genotype is more represented in control group (17.71%) than controls (2.56%). G allele is more represented among marijuana users and A allele in the control group. Statistical analysis showed significant differences in both genotypes distribution and allelic frequencies (*Fisher's exact test* (G) = 0.01; (A) =0.002) (Table 23)

	CTRL Subjects (n=96)	MJ users (n=39)
Genotypes		
Homozygous G/G	78.13%	97.44%
Homozygous A/A	4.17%	0.00%
Heterozygous G/A	17.71%	2.56%
Alleles		
Allele G	86.98%	98.72%
Allele A	13.02%	1.28%

Fisher's exact test (G) =0.01

Fisher's exact test (A) =0.02

Table 23. Genotypes distribution and allelic frequencies relating to the SNP rs1049353, CNR1 gene.

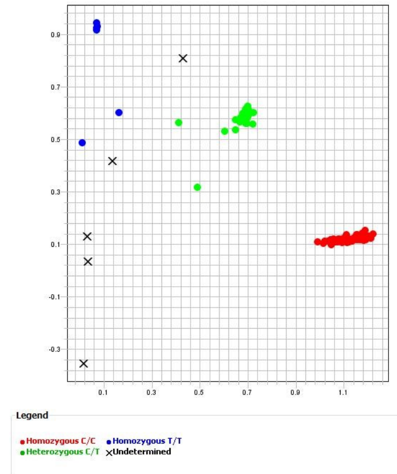


Figure 37. Allelic Discrimination Plot_ rs1049353

SNP rs2180619, CNR1 gene

Genotypes distribution and allelic frequencies relating to the rs2180619 are homogenously distributed between controls and cannabis users. Statistical analysis confirmed this observation (*Fisher's exact test* (G) = 0.9; *Fisher's exact test* (A) = 1) (Table 24).

	CTRL Subjects (n=92)	MJ users (n=38)
Genotypes		
Homozygous A/A	21.74%	23.68%
Homozygous G/G	34.78%	36.84%
Heterozygous A/G	43.48%	39.47%
Alleles		
Allele A	43.48%	43.42%
Allele G	56.52%	56.58%

Fisher's exact test (G) =0.9
Fisher's exact test (A) =1

Table 24. Genotypes distribution and allelic frequencies relating to the SNP rs2180619, CNR1 gene.

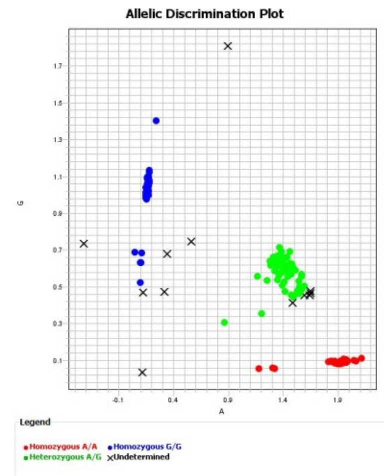


Figure 38. Allelic Discrimination Plot_rs2180619

SNP rs806379, CNR1 gene

Also in the case of rs806379, the genotypes are homogenously distributed, comparing controls and cannabis users. At the allelic level, A allele is slightly more represented in controls (47.40%), compared to marijuana users (42.31%) and T allele frequency is higher in the marijuana users group (57.69%) compare to controls (52.60%). No significant differences have been highlighted with statistical analysis (*Fisher's exact test* (G) = 0.62; *Fisher's exact test* (A) = 0.50) (Table 25).

	CTRL Subjects (n=96)	MJ users (n=39)
Genotypes		
Homozygous A/A	19.79%	17.95%
Homozygous T/T	25.00%	33.33%
Heterozygous A/T	55.21%	48.72%
Alleles		
A allele	47.40%	42.31%
T allele	52.60%	57.69%

Fisher's exact test (G) =0.62
Fisher's exact test (A) =0.5

Table 25. Genotypes distribution and allelic frequencies relating to the SNP rs806379, CNR1 gene.

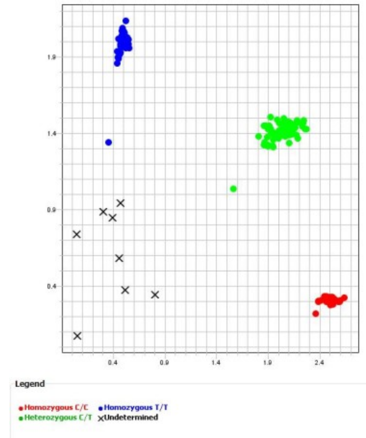


Figure 39. Allelic Discrimination Plot_rs806379

SNP rs6454674, CNR1 gene

For the synonymous substitution rs6454674 in the control group the higher-frequency genotype is the heterozygous G/T (51.06%, compared to 40.00% in cannabis users); in the marijuana users group the higher-frequency genotype is instead homozygous T/T (52.50%, compare to 36.17% in the controls). The larger represented allele is for both groups T allele, with higher frequency among marijuana users (72.50%), than controls (61.70%). G allele appears to be higher in control group (38.30%), compare to marijuana users (27.50%). However, no significant differences have been revealed (*Fisher's exact test* (G) = 0.21; *Fisher's exact test* (A) =0.09) (Table 26).

	CTRL Subjects (n=96)	MJ users (n=39)
Genotypes		
Homozygous G/G	12.77%	7.50%
Homozygous T/T	36.17%	52.50%
Heterozygous G/T	51.06%	40.00%
Alleles		
G allele	38.30%	27.50%
T allele	61.70%	72.50%

Fisher's exact test (G) =0.21
Fisher's exact test (A) =0.09

Table 26. Genotypes distribution and allelic frequencies relating to the SNP rs6454674, CNR1 gene.

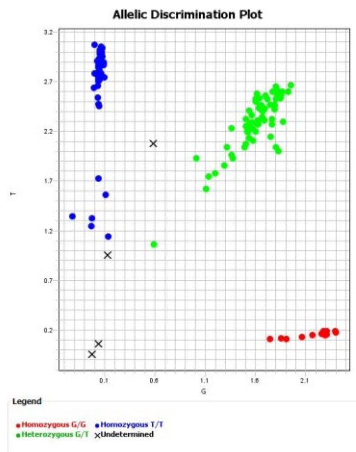


Figure 40. Allelic Discrimination Plot_rs6454674

SNP rs12720071, CNR1 gene

Both genotypes distribution and allelic frequencies relating to the rs12720071, a UTR 3' variant, do not show any differences, comparing the two groups. Homozygous T/T genotype is not represented in the entire population. Statistical analysis is consistent with these observations (*Fisher's exact test* (G) = 1; *Fisher's exact test* (A) = 1) (Table 27).

	CTRL Subjects (n=95)	MJ users (n=39)
Genotypes		
Homozygous C/C	21.88%	20.51%
Homozygous T/T	0.00%	0.00%
Heterozygous C/T	78.13%	79.49%
Alleles		
C allele	60.94%	60.26%
T allele	39.06%	39.74%

Fisher's exact test (G) =1
Fisher's exact test (A) =1

Table 27. Genotypes distribution and allelic frequencies relating to the SNP rs12720071, CNR1 gene.

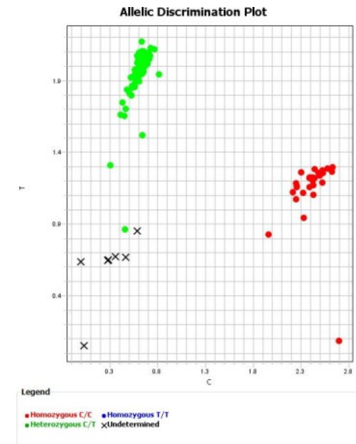


Figure 41. Allelic Discrimination Plot_rs12720071

SNP rs2023239, CNR1 gene

The rs2023239 SNP results in a high distribution of homozygous T/T subjects in the control groups (48.96%), compared to marijuana users (37.50%); homozygous C/C genotype shows similar distributions and heterozygous C/T genotype is more represented among marijuana users (50.00%), than controls (40.63%). T allele is the most frequent allele among all the participants (69.27% in the controls and 62.50% in cannabis users group). No significant differences have been observed with statistical analysis (Fisher's exact test (G) = 0.469; (A) = 0.32) (Table 28).

	CTRL Subjects (n=95)	MJ users (n=39)
Genotypes		
Homozygous C/C	10.42%	12.50%
Homozygous T/T	48.96%	37.50%
Heterozygous C/T	40.63%	50.00%
Alleles		
C allele	30.73%	37.50%
T allele	69.27%	62.50%

Fisher's exact test (G) =0.47
Fisher's exact test (A) =0.32

Table 28. Genotypes distribution and allelic frequencies relating to the SNP rs2023239, CNR1 gene.

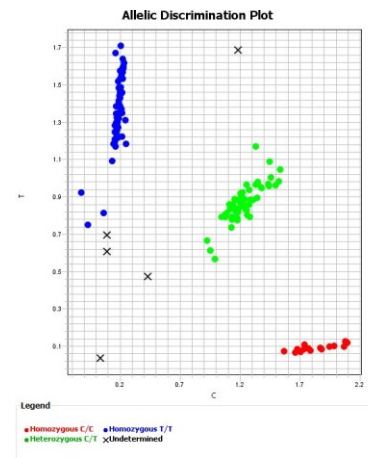


Figure 42. Allelic Discrimination Plot_rs2023239

SNP rs2501431, CNR2 gene

Heterozygous A/G genotype carriers are homogenously distributed in the two groups with a frequency of 41%. Homozygous A/A genotype is instead more frequent in the marijuana group (58.97%), compared to controls (47.37%). Homozygous G/G genotype is not represented among marijuana users; in the control group is the genotype with the lower frequency (11.58%). The most frequent allele is A allele, that is larger represented among cannabis users (79.49%), than controls (67.89%). Statistical analysis results in differences concerning genotypes distribution (*Fisher's exact test* (G) = 0.058); no significant differences have been reported in the allele frequency analysis (*Fisher's exact test* (A) = 0.07) (Table 29).

	CTRL Subjects (n=95)	**MJ users** (n=39)
Genotypes		
Homozygous A/A	47.37%	58.97%
Homozygous G/G	11.58%	0.00%
Heterozygous A/G	41.05%	41.03%
Alleles		
G allele	32.11%	20.51%
A allele	67.89%	79.49%

Fisher's exact test (G) =0.058
Fisher's exact test (A) =0.07

Table 29. Genotypes distribution and allelic frequencies relating to the SNP rs2501431, CNR2 gene.

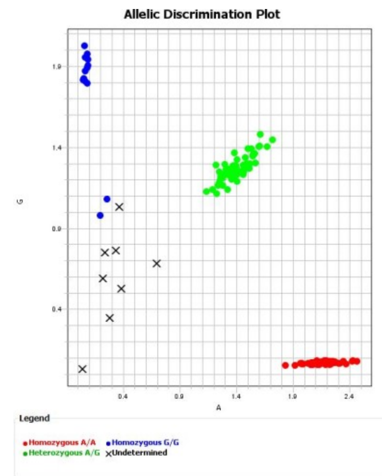


Figure 43. Allelic Discrimination Plot_rs2501431

Haplotypes analysis

PLINK analysis was conducted by the sliding window method and by the haplotype based case/control association. The SNPs involved in haplotypes analysis for American population were the 6 SNPs of CNR1 on chromosome 6 (rs1049353|rs806379|rs6454674|rs2023239|rs12720071|rs2180619).
Positive associations were found at the 2-4 SNPs levels (Table 30).

Sliding win.	SNPS forming the haplotype	Haplotype	Frequency in MJ	Frequency in CTRLs	Test for association CHISQ	Degree of freedom	P value
2 SNPs	rs12720071|rs1049353	TT	0,009868	0,08497	5,279	1	0,02159
	rs1049353|rs2023239	TT	0,00692	0,09218	6,402	1	0,0114
3 SNPs	rs12720071|rs1049353|rs2023239	TTT	0,004799	0,0684	4,721	1	0,02979
	rs1049353|rs2023239|rs806379	TTA	0,008201	0,09034	6,019	1	0,01415
4 SNPs	rs12720071|rs1049353|rs2023239|rs806379	TTTA	0,006228	0,07111	4,705	1	0,03007
	rs1049353|rs2023239|rs806379|rs6454674	TTAT	0,009029	0,07984	4,907	1	0,02674
	rs1049353|rs2023239|rs806379|rs6454674	CCTT	0,3776	0,2373	5,339	1	0,02086

Table 30. The significant associations are reported after application of the sliding window method using 2-5 or 6 marker windows. Only 2, 3 and 4 SNPs positive associations are shown.

4.4 ENVIRONMENTAL STUDY IN THE AMERICAN POPULATION

Logistic regression was used to assess the association between demographic and genetic variables on marijuana use. The first model evaluated the effects of 4 independent variables (gender, marital status, BMI, educational level) on the dependent variable, marijuana use (Table 31). All four variables were entered into the model in this initial step: gender (male vs. female), education (divided in four categories: 1.Some High School/GED, 2. High School Diploma, 3.Some College, 4. College Graduate, Masters, Ph.D), BMI (≥25 vs. <25), and marital status (married vs. not married) (See Table 10 for data count).

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Education	-.573	.217	6.967	1	.008	.564
	Gender (1)	1.575	.460	11.726	1	.001	4.830
	Marital Status (1)	1.103	.865	1.626	1	.202	3.012
	BMI (1)	.161	.441	.133	1	.715	1.175
	Constant	-1.414	1.014	1.947	1	.163	.243
Step 2[a]	Education	-.561	.215	6.832	1	.009	.571
	Gender (1)	1.605	.452	12.586	1	.000	4.978
	Marital Status (1)	1.102	.866	1.619	1	.203	3.010
	Constant	-1.391	1.010	1.896	1	.169	.249
Step 3[a]	Education	-.546	.212	6.636	1	.010	.579
	Gender (1)	1.592	.449	12.584	1	.000	**4.914**
	Constant	-.406	.636	.407	1	.523	.666

a. Variable(s) entered on step 1: Education, Gender, Marital status, BMI.
b. Variable(s) removed on step 2: BMI.
c. Variable(s) removed on step 3: Marital status.

Table 31. Logistic regression model 1.
Independent variables: gender, marital status, BMI, educational level; dependent variable: MJ use.

A backwards elimination procedure was used, in which one non-significant variable was removed at a time until only statistically significant ($p<0.05$) predictors remained. BMI was removed in step 2 and marital status in step 3. The last step (step 3) shows the final model with only two significant variables remaining: education and gender. Gender was used as a categorical variable with male participants being almost five times as likely to be marijuana users compared to females (OR=4.91, $p<0.0001$). Education was used as a numeric variable: the significant difference highlighted in the model means that for each increase of one category of education, the risk of marijuana use drops by approximatively half (OR = 0.579).

The second logistic regression model aimed to determine the demographic and genetic correlates of marijuana use (Table 32). This model included all four demographic variables (gender, marital status, BMI, and education) as well as eight SNP allele types as independent variables.
The database was set up so that each SNP (e.g. rs1800497 ANKK1) was a separate variable, and there were two rows per participant containing the two alleles for that SNP (e.g. genotype AG would have A in the first row and G in the second row). Generalized Estimating Equations (GEE) were used to account for within-subject correlations; an exchangeable correlation matrix was specified. The GEE procedures were run using SAS version 9.4 (SAS Institute, Cary NC); all other statistical analyses were conducted using IBM SPSS (version 24).

Parameter	Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept	1.2574	0.9034	-0.5132	3.0280	1.39	0.1640
rs1800497ANKK1_A allele	-0.0001	0.0001	-0.0002	0.0001	-0.77	0.4393
_G allele	0.0000	0.0000	0.0000	0.0000	.	.
rs1049353 CNR1_C allele	0.0005	0.0001	0.0002	0.0008	3.58	0.0003
_T allele	0.0000	0.0000	0.0000	0.0000	.	.
rs806379 CNR1_A allele	-0.0001	0.0001	-0.0003	0.0001	-1.08	0.2804
_T allele	0.0000	0.0000	0.0000	0.0000	.	.
rs6454674 CNR1_G allele	-0.0001	0.0001	-0.0002	0.0001	-1.07	0.2841
_T allele	0.0000	0.0000	0.0000	0.0000	.	.
rs2023239 CNR1_C allele	0.0001	0.0001	-0.0001	0.0003	0.85	0.3965
_T allele	0.0000	0.0000	0.0000	0.0000	.	.
rs12720071 CNR1_C allele	-0.0000	0.0000	-0.0001	0.0001	-0.59	0.5524
_T allele	0.0000	0.0000	0.0000	0.0000	.	.
rs2180619 CNR1_A allele	-0.0000	0.0001	-0.0002	0.0002	-0.16	0.8725
_G allele	0.0000	0.0000	0.0000	0.0000	.	.
rs2501431 CNR2_A allele	0.0001	0.0001	-0.0000	0.0003	1.77	0.0762
_G allele	0.0000	0.0000	0.0000	0.0000	.	.
Gender	-1.7132	0.4739	-2.6421	-0.7843	-3.61	0.0003
Marital Status	-1.0282	0.7983	-2.5928	0.5364	-1.29	0.1978
Education	-0.5327	0.2089	-0.9421	-0.1232	-2.55	0.0108
BMI	0.0213	0.4518	-0.8642	0.9069	0.05	0.9623

Table 32. Logistic regression model 2. Independent variables: gender, marital status, BMI, educational level, genotypes; dependent variable: MJ use.

As shown in the table above, rs1049353 SNP of CNR1 is a statistically significant risk factor for cannabis use (p=0.0003), after adjusting for demographic variables. Also, gender and education remained significant risk factors for marijuana use in this model.

4.5 EPIGENETIC STUDY

This study is conducted on the American population at the National Institute on Drug Abuse (NIDA), Baltimore, MD, USA.
To highlight epigenetic differences between marijuana users and control subjects, DNA methylation at specific loci of ANKK1, DRD2, CNR1, NCAM1 genes has been analysed through DNA immunoprecipitation (MeDIP) technique, followed by quantitative Real Time PCR.

DNA methylation status was found significantly higher in marijuana users compared to control subjects in two of the regions analysed (Table 33): the first site is located in the exon 8 of DRD2 gene at +66.7 kb from the transcription start site (TSS) (p 0.034); the second is located on a CpG region at +3 kb from the TSS in the NCAM1 gene (p 0.0004). An increase in DNA methylation status can be observed also for ANKK1 gene, the region analysed is at -0.25 kb from the TSS and in another site of DRD2 gene, on a CpG region at +0.9 kb from the TSS, but statistical analysis did not confirm significant differences.
No differences in DNA methylation were found at DRD2 -0.4 kb, NCAM1 +0.4 kb, CNR1 +22.31 kb comparing marijuana users and control subjects.

Gene	Site of DNA methylation quantification	P Value (MJ users vs CTRL subjects)
ANKK1	-0.25 TSS	0.2054
DRD2	-0.4 kb TSS	0.6086
DRD2	+0.9 kb TSS CpG	0.1380
DRD2	+66.7 kb exon8	0.034
CNR1	+22.31 kb SNP region (rs1049353)	0.8535
NCAM1	+0.4 kb TSS	0.9536
NCAM1	+3 kb TSS CpG	0.0004

Table 33. List of the regions where DNA methylation level has been quantified, with related p value, from the comparison between marijuana users and controls.

Details on the primer position in the genome from UCSC browser and statistical analysis outcome, for each analysed site, are reported in the following pages (pages 71-76; Figures 44-50).

ANKK1, *Ankyrin Repeat and Kinase Domain Containing 1*gene, -0.25 kb TSS

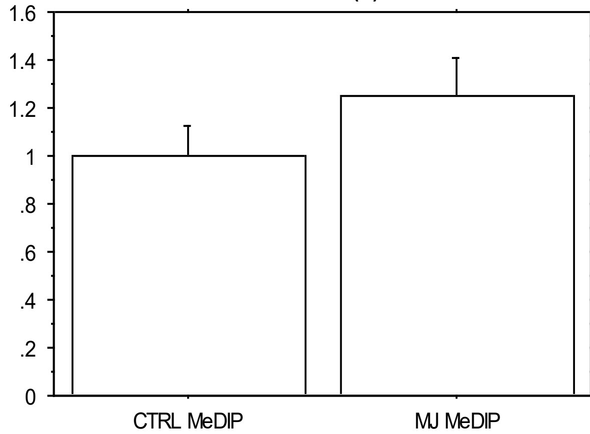
ANOVA Table for ANKK1 (-0.25 kb)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	1.356	1.356	1.627	.2056	1.627	.229
Residual	86	71.717	.834				

Means Table for ANKK1 (-0.25 kb)
Effect: Treatment

	Count	Mean	Std. Dev.	Std. Err.
CTRL MeDIP	52	1.000	.901	.125
MJ MeDIP	36	1.252	.930	.155

Interaction Bar Plot for ANKK1 (-0.25 kb)
Effect: Treatment
Error Bars: ± 1 Standard Error(s)



Unpaired t-test for ANKK1 (-0.25 kb)
Grouping Variable: Treatment
Hypothesized Difference = 0

	Mean Diff.	DF	t-Value	P-Value
CTRL MeDIP, MJ MeDIP	-.253	86	-1.275	.2056

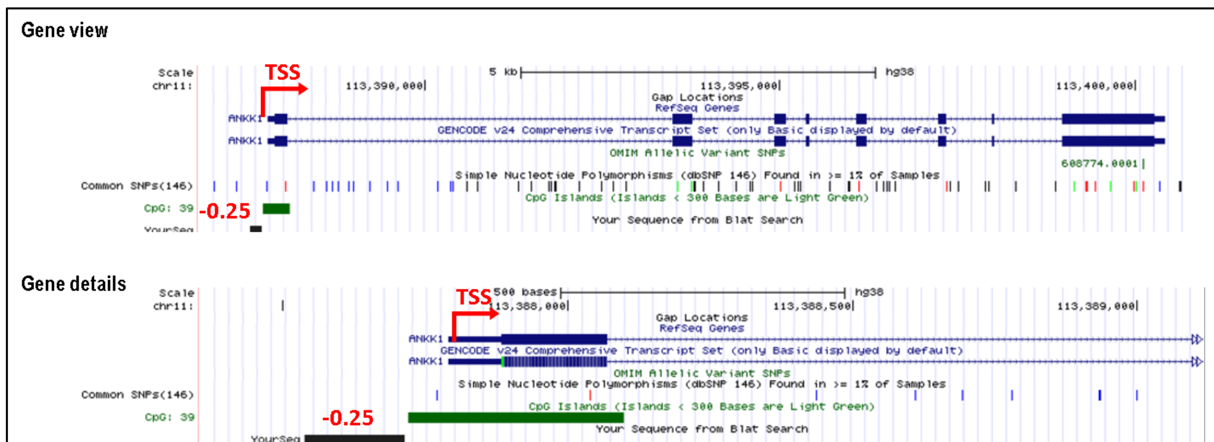


Figure 44. Primers annealing region, -0.25 kb from TSS, ANKK1 gene (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly)

DRD2, *Dopamine Receptor2*, -0.4 kb TSS

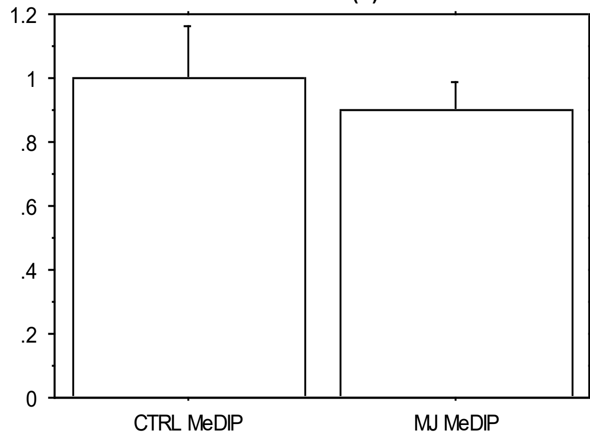
ANOVA Table for DRD2 (-0.4 kb)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	.198	.198	.268	.6064	.268	.079
Residual	75	55.552	.741				

Means Table for DRD2 (-0.4 kb)
Effect: Treatment

	Count	Mean	Std. Dev.	Std. Err.
CTRL MeDIP	43	1.000	1.054	.161
MJ MeDIP	34	.898	.518	.089

Interaction Bar Plot for DRD2 (-0.4 kb)
Effect: Treatment
Error Bars: ± 1 Standard Error(s)



Unpaired t-test for DRD2 (-0.4 kb)
Grouping Variable: Treatment
Hypothesized Difference = 0

	Mean Diff.	DF	t-Value	P-Value
CTRL MeDIP, MJ MeDIP	.102	75	.517	.6064

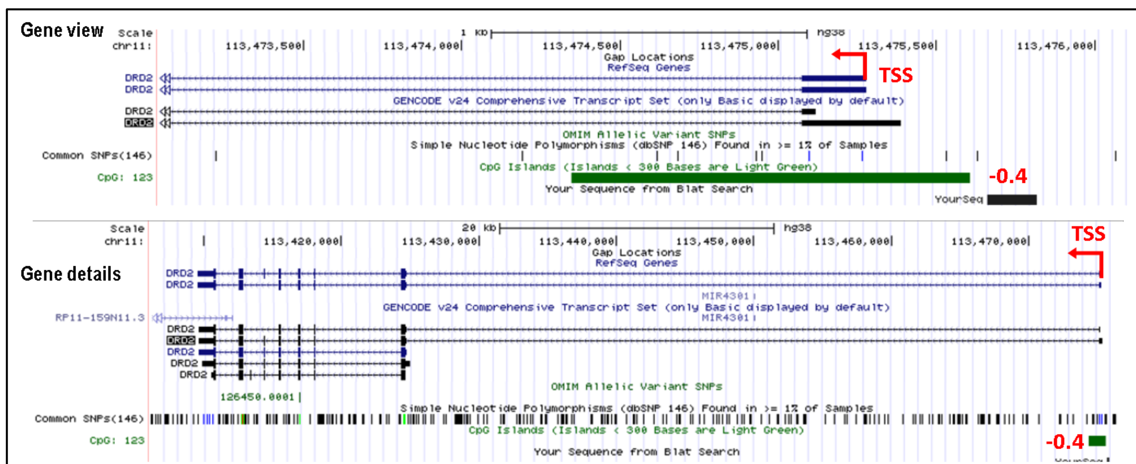


Figure 45. Primers annealing region, -0.4 kb from TSS, DRD2 gene (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly)

DRD2, *Dopamine Receptor2*, +0.9 kb TSS on CpG

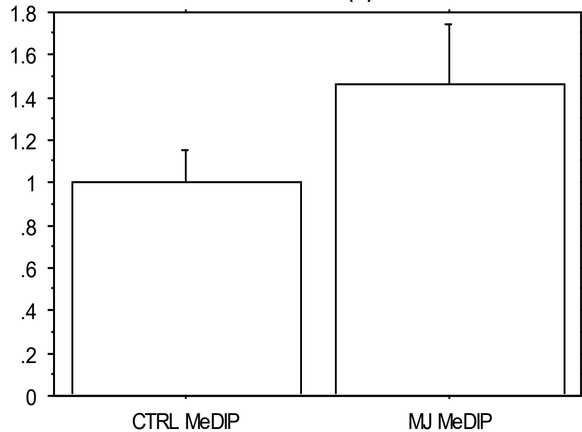
ANOVA Table for DRD2 (+0.9kb)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	3.514	3.514	2.254	.1381	2.254	.299
Residual	66	102.908	1.559				

Means Table for DRD2 (+0.9kb)
Effect: Treatment

	Count	Mean	Std. Dev.	Std. Err.
CTRL MeDIP	38	1.000	.935	.152
MJ MeDIP	30	1.458	1.560	.285

Interaction Bar Plot for DRD2 (+0.9kb)
Effect: Treatment
Error Bars: ± 1 Standard Error(s)



Unpaired t-test for DRD2 (+0.9kb)
Grouping Variable: Treatment
Hypothesized Difference = 0

	Mean Diff.	DF	t-Value	P-Value
CTRL MeDIP, MJ MeDIP	-.458	66	-1.501	.1381

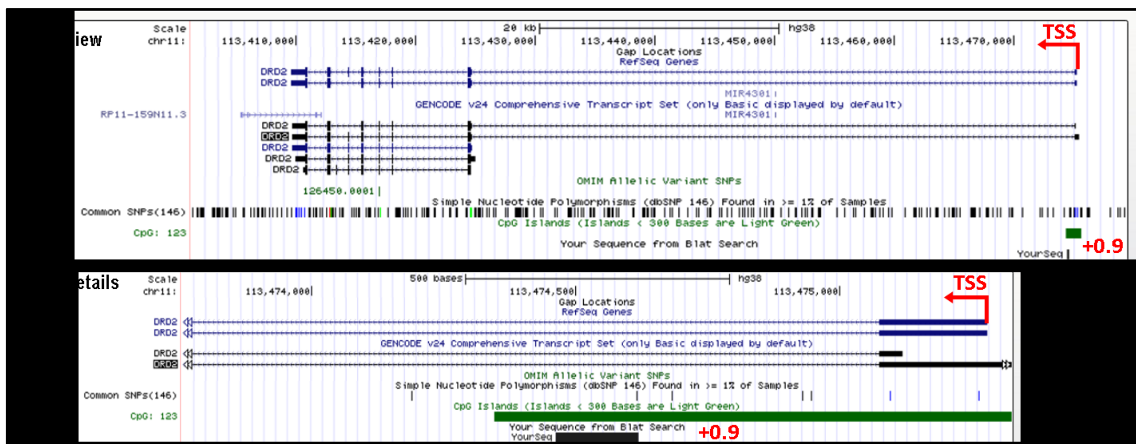


Figure 46. Primers annealing region, +0.9 kb from TSS, DRD2 gene (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly)

DRD2, *Dopamine Receptor2*, +66.7 kbTSS

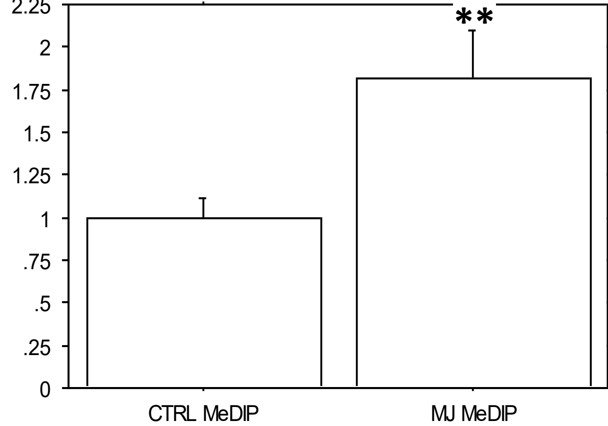
ANOVA Table for DRD2 (66.7kb)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	14.729	14.729	9.084	.0034	9.084	.863
Residual	88	142.683	1.621				

Means Table for DRD2 (66.7kb)
Effect: Treatment

	Count	Mean	Std. Dev.	Std. Err.
CTRL MeDIP	53	1.000	.851	.117
MJ MeDIP	37	1.822	1.708	.281

Interaction Bar Plot for DRD2 (66.7kb)
Effect: Treatment
Error Bars: ± 1 Standard Error(s)



Unpaired t-test for DRD2 (66.7kb)
Grouping Variable: Treatment
Hypothesized Difference = 0

	Mean Diff.	DF	t-Value	P-Value
CTRL MeDIP, MJ MeDIP	-.822	88	-3.014	.0034

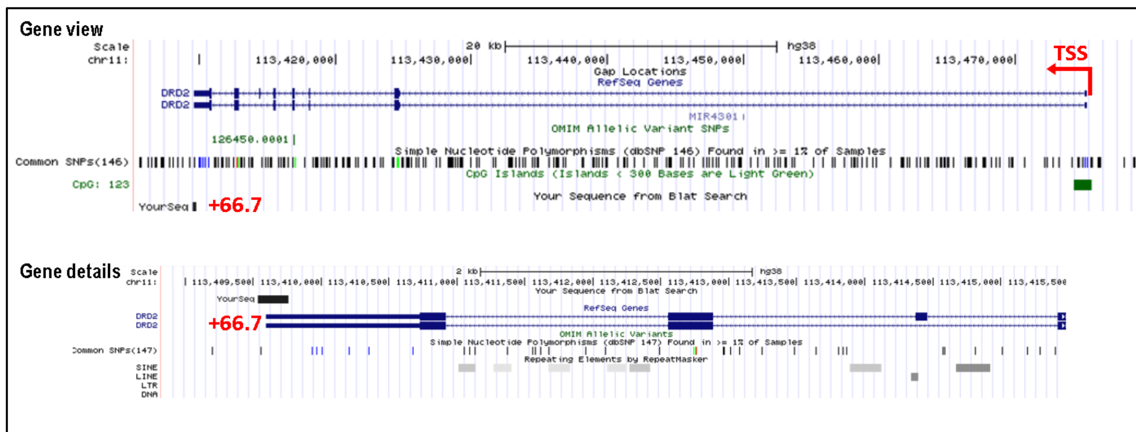


Figure 47. Primers annealing region, +66.7 kb from TSS, DRD2 gene (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly)

NCAM1, *Neural Cell Adhesion Molecule 1*, +0.4 kbTSS

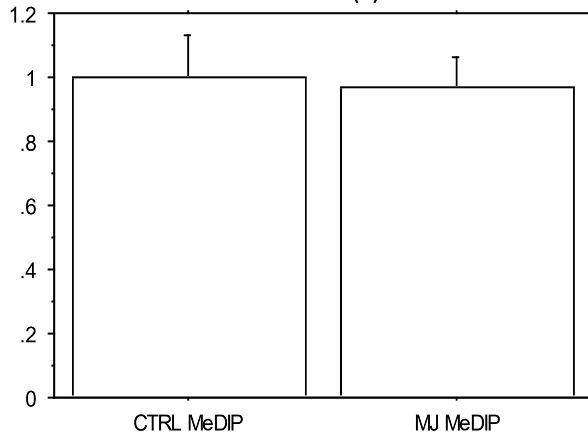
ANOVA Table for NCAM1 (+0.4kb)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	.018	.018	.035	.8531	.035	.054
Residual	64	32.572	.509				

Means Table for NCAM1 (+0.4kb)
Effect: Treatment

	Count	Mean	Std. Dev.	Std. Err.
CTRL MeDIP	40	1.000	.823	.130
MJ MeDIP	26	.967	.495	.097

Interaction Bar Plot for NCAM1 (+0.4kb)
Effect: Treatment
Error Bars: ± 1 Standard Error(s)



Unpaired t-test for NCAM1 (+0.4kb)
Grouping Variable: Treatment
Hypothesized Difference = 0

	Mean Diff.	DF	t-Value	P-Value
CTRL MeDIP, MJ MeDIP	.033	64	.186	.8531

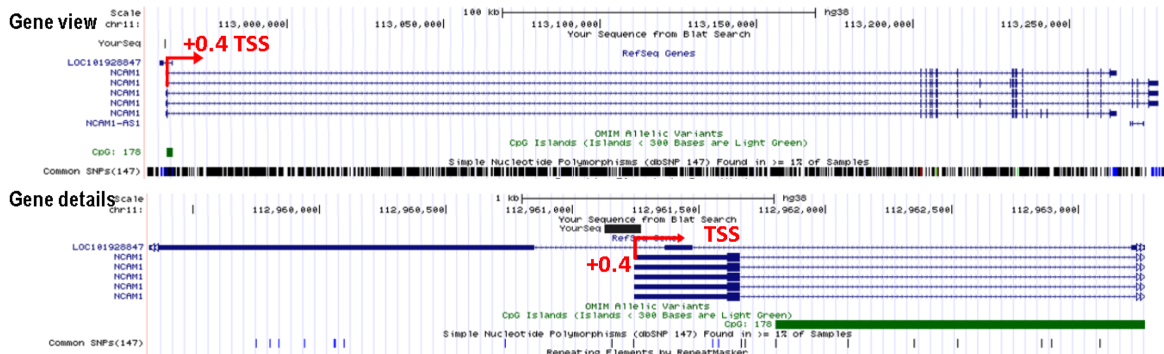


Figure 48. Primers annealing region, +0.4 kb from TSS, NCAM1 gene (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly)

NCAM1, *Neural Cell Adhesion Molecule 1*, +3 kb TSS CpG

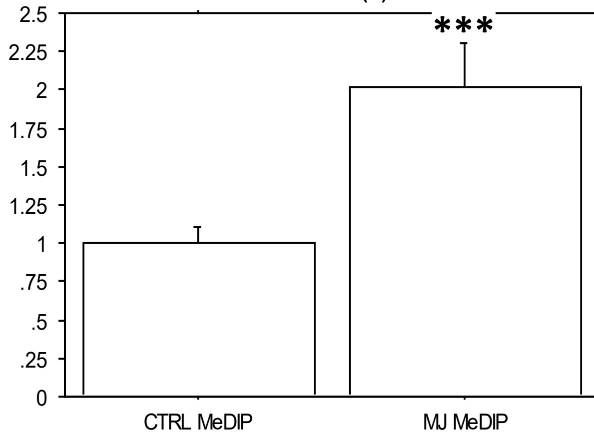
ANOVA Table for NCAM1 (+3.0kb)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	22.246	22.246	14.529	.0003	14.529	.979
Residual	85	130.146	1.531				

Means Table for NCAM1 (+3.0kb)
Effect: Treatment

	Count	Mean	Std. Dev.	Std. Err.
CTRL MeDIP	50	1.000	.770	.109
MJ MeDIP	37	2.023	1.676	.275

Interaction Bar Plot for NCAM1 (+3.0kb)
Effect: Treatment
Error Bars: ± 1 Standard Error(s)



Unpaired t-test for NCAM1 (+3.0kb)
Grouping Variable: Treatment
Hypothesized Difference = 0

	Mean Diff.	DF	t-Value	P-Value
CTRL MeDIP, MJ MeDIP	-1.023	85	-3.812	.0003

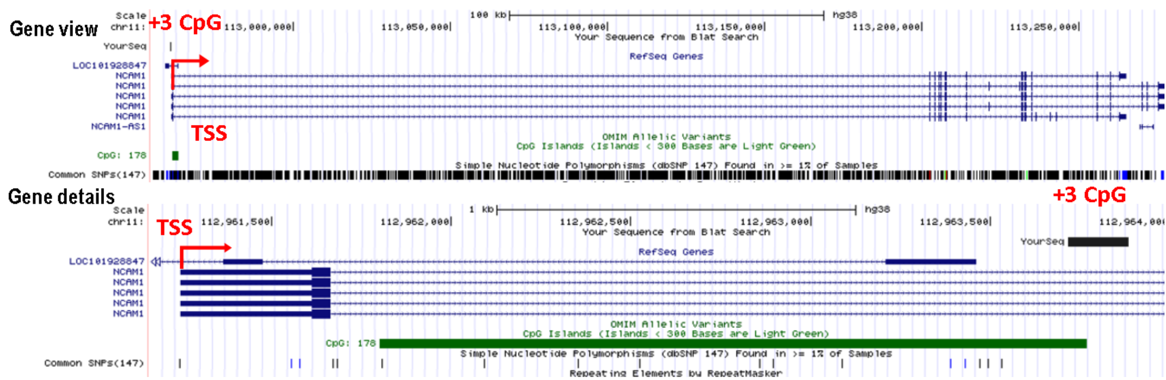


Figure 49. Primers annealing region, +3 kb from TSS, NCAM1 gene (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly)

CNR1, *Cannabinoid Receptor 1*, +22.31 kbTSS rs1049353 SNP

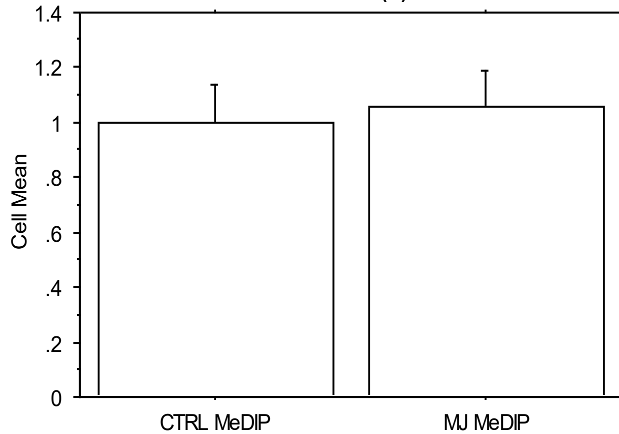
ANOVA Table for CNR1 (22.3kb)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Pow er
Treatment	1	.063	.063	.084	.7726	.084	.059
Residual	78	58.208	.746				

Means Table for CNR1 (22.3kb)
Effect: Treatment

	Count	Mean	Std. Dev.	Std. Err.
CTRL MeDIP	47	1.000	.923	.135
MJ MeDIP	33	1.057	.771	.134

Interaction Bar Plot for CNR1 (22.3kb)
Effect: Treatment
Error Bars: ± 1 Standard Error(s)



Unpaired t-test for CNR1 (22.3kb)
Grouping Variable: Treatment
Hypothesized Difference = 0

	Mean Diff.	DF	t-Value	P-Value
CTRL MeDIP, MJ MeDIP	-.057	78	-.290	.7726

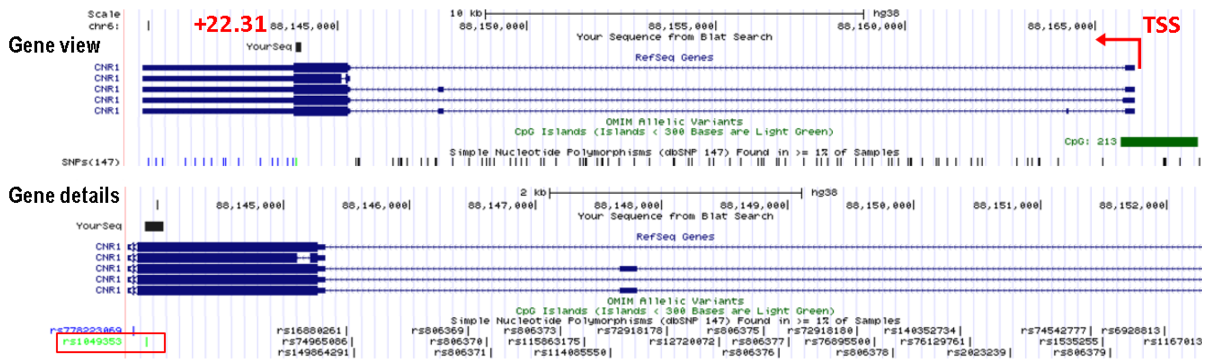


Figure 50. Primers annealing region, +22.31 kb from TSS, CNR1 gene (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly)

5. Discussion

The study aim was to investigate genetic, environmental and epigenetic risk factors of cannabis use disorders.

Genetic factors

The candidate gene association study conducted in Caucasian population, comparing genomic DNA of 93 controls and 92 marijuana users, has shown two potential risk factors for cannabis use disorders: the SNP rs1800497 (Taq1A) of ANKK1 gene and the SNP rs1049353 (G1359A) of CNR1 gene. Taq1A allele (rs1800497) is associated with a reduced D2 receptor density in the brain (Pohjalainen et al., 1998, Jönsson et al., 1999) and a lower D2 receptor binding potential in healthy carriers of the minor allele A1 (Lys713) (Gluskin et al., 2016). In our study, we found that the SNP rs1800497 was significantly higher in Caucasian population in the marijuana group compared to controls ($p < 0.034$). This result is also reflected in the genotypic distribution, where heterozygous T/C (thymine/cytosine; A1/A2) was more frequent in the marijuana users ($p < 0.032$). C (A2) allele and homozygous CC (A2/A2) genotype were represented most in the control group (Figure 51).

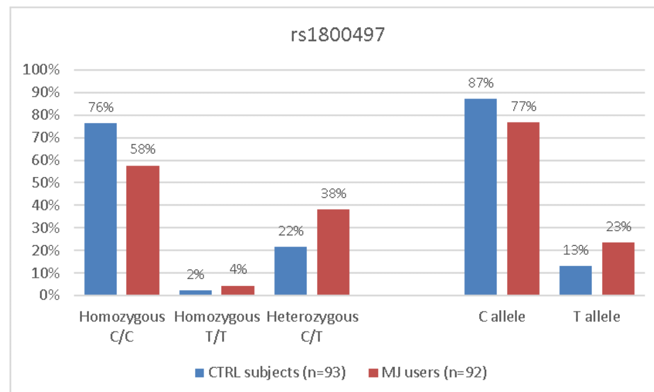


Figure 51. Genotypic distribution and allelic frequencies relative to the rs1800497 SNP, comparing MJ users (red) and CTRL subjects (blue).

The TaqIA SNP is the most studied genetic variant in drug addictions (Ponce et al. 2009). In a preliminary study, participants with the T allele of rs1800497, reflecting lower D2 receptor density, reported higher subjective effects in response to acute cocaine administration, than those with genotypes associated with higher D2 receptor density. These individuals may have increased vulnerability to continue using cocaine or they may be at greater risk to relapse (Spellicy et al., 2014). Taq1A allele has also been associated with conduct disorder, problematic alcohol use in adolescents (Esposito-Smythers et al., 2009; Blum et al., 1999) and defined as a risk factor for alcoholism (Agrawal et al. 2012) and cannabinoid dependence (Nacak et al., 2012). These observations are consistent with our results, which confirm a protective role of A2 allele (Glu) in marijuana use and A1 allele (Lys) as one of the risk factor for cannabis use disorders development. It is interesting focusing on the position of ANKK1 gene on the chromosome 11 and the hypothesized role of this kinase, highly expressed in the brain (Neville et al., 2004). This SNP is located in the ANKK1 exon9, 10541 bp downstream of the termination codon of the DRD2 gene (Ponce et al., 2009) (Figure 52).

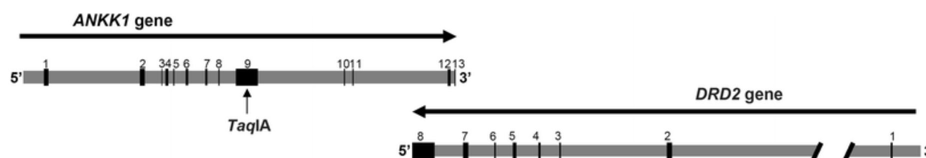


Figure 52. ANKK1 and DRD2 position on chromosome 11. The genes overlap at their 3′ends. The TaqIA polymorphism occurs in the exon 9 of ANKK1. The arrows indicate the open reading frame (ORF). Exons are shown by black boxes (Ponce et al., 2009)

Not all studies confirm similar association; for example, in another study, TaqIA polymorphism resulted associated with opioid dependence risk, but not with stimulants or marijuana dependence (Deng et al., 2015).

The predicted *ankyrin repeat and kinase domain containing 1* protein has a serine/threonine kinase domain and an ankirin repeat domain with 11 repeated sequences, generally involved in protein-protein interactions (Figure 53). The protein has been thus related to the receptor interacting protein (RIP) kinases family that participate in signal transduction pathways and are closely related to members of the interleukin-1 receptor-associated kinases (IRAKs) (Meylan & Tschopp 2005).

This pathway mediates the response of eukaryotic cells to external stimuli such as infection, inflammation, cellular differentiation programs, and DNA damage, activating transcription factors or stimulation of cell death (Meylan & Tschopp 2005). The variation in Lys (K) residue position of the TaqIA polymorphism in the ankirin repeat domain could profoundly alter the protein interactions.

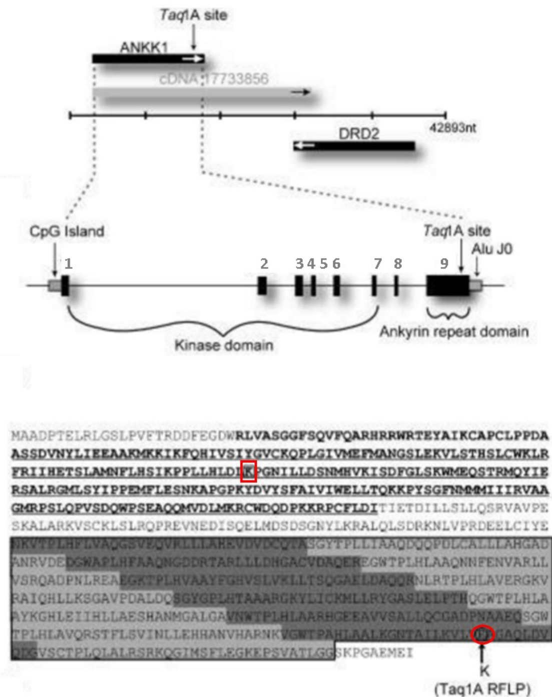


Figure 53. Amino acid sequence of Ankk1 protein is reported: the kinase domain (highlighted in bold text and underlined) with the key lysine residue characteristic of a serine/threonine kinase domain (red square box) and an ankirin repeat domain (boxed, alternate repeat units are highlighted in light and dark gray), with the red circled residue of Taq1A (rs1800497) (Neville et al., 2004)

For many years, TaqIA polymorphism was incorrectly thought to belong to the flanking DRD2 gene (Figure 53) and was studied as a marker of DRD2 gene itself (Ponce et al., 2009). Studies show that ANKK1 mRNA can be upregulated after activation of D1R-like dopamine receptors with apomorphine, a dopaminergic agonist, in mouse astrocytes (Hoenicka et al., 2010) and in contrast, D2R-like agonists 7-OH-DPAT (7-Hydroxy-DPAT) and aripiprazole caused a significant downregulation of ANKK1 mRNA (Ponce et al., 2016). At protein level, D2R-like agonist 7-OH-DPAT caused a significant increment of ANKK1 protein in the mouse striatal tissue compared to the prefrontal cortex (Ponce et al., 2016). The activation of D1R-like and D2R-like leads to opposite transcriptional regulation of ANKK1 by specific pathways, as PKA and PLC pathways (Ponce et al., 2016). All these observations are consistent with a potential connection between ANKK1 and the dopaminergic system functioning, suggesting Ankk1 protein may have its own role to play in this pathway and not only in association with D2R variation.

Further studies are needed to clarify the molecular mechanisms by which ANKK1 could be associated with the dopaminergic pathway and how ANKK1 polymorphic alleles would impact cannabis use disorders and in general addictions vulnerability.

The second genetic risk factor, identified in both Caucasian and American population, was the SNP rs1049353 (G1539A), a synonymous substitution of a guanine to adenine at nucleotide position 1359 of cannabinoid receptor 1 gene (CNR1). Consistently with Hardy Weinberg equilibrium, G allele and homozygous GG were respectively found as the more frequent allele and genotype in both Caucasian and American population (Table 34).

rs1049353	Hardy–Weinberg equilibrium		
	TT (pxp) (AA)	CC (qxq) (GG)	TC(2xpxq) (GA)
European American	0.27093x0.27093=0.0734: **7%**	0.72907x0.72907= 0.5256376: **52.56%**	0.0771636: **39.5%**
African American	0.071266x0.071266=0.0051: **0.5%**	0.928734x0.928734= 0.86255: **86.25%**	0.0086: **13.24%**

Table 34. Hardy Weinberg frequency calculation. MAF index provided by NHLBI Exome Sequencing Project (ESP); (Exome Variant Server) http://evs.gs.washington.edu/EVS/. Genotypes are reported as TT/CC/TC because rs1049353 allele is reverse to the genome, according to NCBI report.

Heterozygous GA has a higher frequency in Caucasian population, compared to American population. G allele may instead represent a risk factor for cannabis use disorders in American population, since G allele ($p<0.002$) and homozygous GG genotype ($p<0.01$) were found significantly higher in marijuana users compared to control subjects (Figure 54). In Caucasian population, heterozygous G/A carriers were found more frequent among marijuana users than controls ($p< 0.051$), but no significant differences were observed at allelic level.

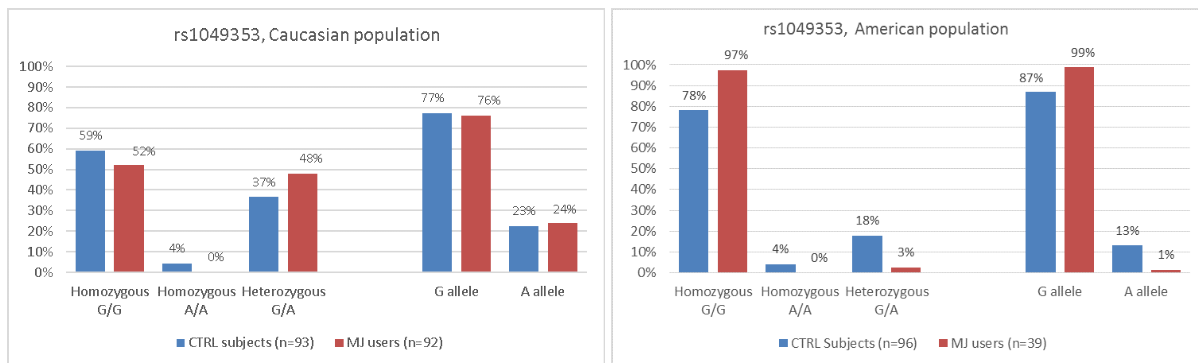


Figure 54. Genotypic distribution and allelic frequencies relative to the rs1049353 SNP, comparing MJ users (red) and CTRL subjects (blue) in Caucasian and American population.

This result is consistent with the finding that a nominal association was found between the G allele, SNP rs1049353, and existence of one or more cannabis dependence symptoms (Hartman et al., 2009). In addition, G allele of rs1049353 was also found in a haplotype, which might confer a protective effect against alcohol dependence, together with T allele of rs6454674 and rs806368 (Marcos et al., 2012). Conversely, few studies reported the opposite effect of rs1049353 genotype and allele of that found in this study. For example, homozygous A/A genotype was associated with vulnerability to alcohol withdrawal delirium (Schmidt et al., 2002) or rs1049353 A allele in association with AA genotype of rs806379 enhanced impulsivity (Buchmann et al., 2015).

As already stated, variation in the CNR1 gene may be associated with cannabis dependence, but these and other results are in need of replication to clarify the role of this gene in the development of substance use disorders (Hartman et al., 2009).

Furthermore, it is very important to highlight that even silent mutation (those that do not affect amino acid sequence), as the rs1049353 SNP, might affect mRNA conformation and consequently protein folding and function (Komar AA, 2007). For example, DRD2 mRNA folding structure is influenced by T allele of rs6277 that altered the predicted mRNA folding, leading to distinctly decreased DRD2 mRNA stability and translation, and dramatically changed dopamine-induced upregulation of DRD2 expression; the occurrence simultaneously of this SNP with 1101A mutation annulled this effect (Duan et al., 2003). Kimchi-Sarfaty and colleagues (2007) has demonstrated that naturally occurring silent mutations can alter the primary structure of a protein, modifying *in vivo* protein folding and, consequently, function: the substrate specificity of P-glycoprotein, the product of the multidrug resistance 1 (MDR1) gene, was found altered by synonymous SNPs. In addition to altered mRNA conformations, another explanation of this "silent but not invisible" effect (Komar AA, 2007) could be derived from the observation that codon usage is not random, but could be an affect from many factors, such as gene length, location on the chromosome and secondary structure elements (Supek & Vlahovicek, 2005). It has indeed been hypothesized silent mutations, which change frequent codons into rare codons, alter the timing of folding and subsequently protein function (Kimchi-Sarfaty et al., 2007) (Figure 56).

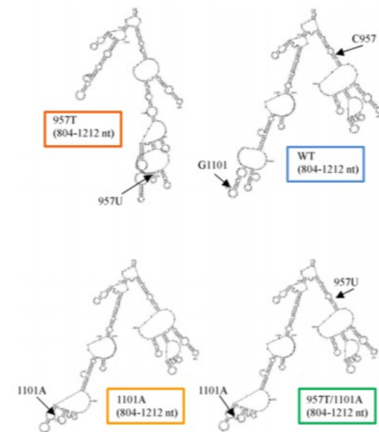


Figure 55. Examples of DRD2 mRNA folding structures predicted by MFOLD (nt 804–1212 of the coding sequence) (Duan et al., 2003).

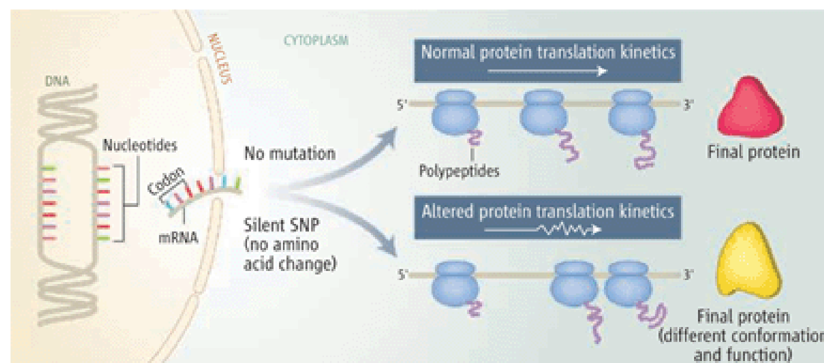


Figure 56. Codon substitution may lead to different kinetics of mRNA (protein) translation, thus yielding a protein with a different final structure and function (Komar AA, 2007).

Interestingly, Isir and colleagues (2016) have recently hypothesized the interactions between CB1 and D2R proteins in marijuana abusers which may be due to the overlapping expressions of both CB1 and D2R (Isir et al., 2016). In addition, they even suggested that the different polymorphisms of CNR1 and DRD2 genes (referring to ANKK1 Taq1A SNP) may play a role in these interactions, promoting or alleviating cannabis use disorder risk factors (Isir et al., 2016). However, the mechanism of interaction is still unclear, but it occurs when both receptors are overexpressed (Mackie, 2005), and overexpression occurs in disease states and during drug abuse (Isir et al., 2016).

Differences were observed in the genotypes distribution ($p<0.058$) for the SNP rs2501431, CNR2 gene (Figure 57). Few studies focus on variations in CNR2 gene and some of them have investigated the role of rs2501431. Study on osteoporosis susceptibility found that rs2501431 was significantly associated with BMD and osteoporosis (Zhang et al., 2015). Other study focus on rs2501431 primary anxiety disorder and depression (Lester et al., 2016). AA homozygous carriers were found with more severe type of depression (Mitjans et al., 2012). In our study, homozygous A/A genotype were found more frequent in the marijuana group compared to controls, but no high differences have been observed in the allelic frequency. This result encourages further studies to investigate variations in CNR2 gene and addiction development.

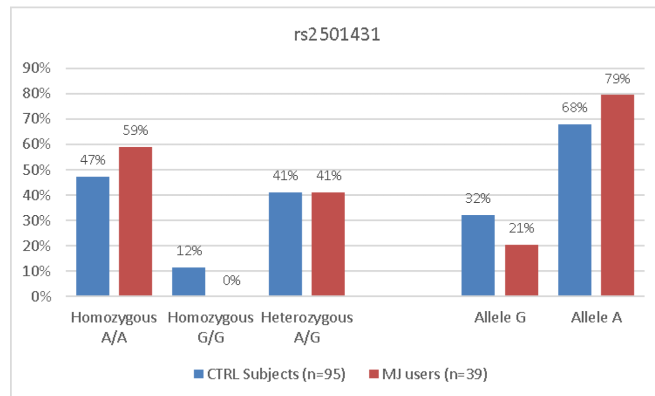


Figure 57. Genotypic distribution and allelic frequencies relative to the rs2501431 SNP, comparing MJ users (red) and CTRL subjects (blue) in American population.

rs2501431	Hardy–Weinberg equilibrium		
	AA (pxp)	GG (qxq)	AG(2xpxq) (GA)
European American	0.573837x0.573837=0.3292889: **32.93%**	0.426163x0.426163=0.1816149: **18.16%**	0.48909: **50%**
African American	0.751248x 0.751248= 0.56437: **56.4%**	0.248752x 0.248752=0.061877: **6%**	0.37375: **37.4%**

Table 35. Hardy Weinberg frequency calculation. MAF index provided by NHLBI Exome Sequencing Project (ESP) for rs2501431; (Exome Variant Server) http://evs.gs.washington.edu/EVS/.

Haplotype based association analysis increases the power to detect genetic traits associated with complex disorders (Marcos et al., 2012), and replication at haplotype level may be more reliable than replication at SNP level (Neale and Sham, 2004; Sullivan, 2007). For this reason it is necessary evaluate if specific set of SNPs on one chromosome tend to occur together, especially in multifactorial traits as cannabis use disorders.

No significant association in Caucasian haplotypes analysis were found (the analysis involved 2 SNPs of chromosome 11 rs1800497|rs6277, ANKK1 and DRD2 genes respectively). For American population we found different combination of SNPs in CNR1 showed significant association with marijuana use; haplotypes analysis involved the 6 SNPs of CNR1 on chromosome 6 (rs1049353| rs806379| rs6454674| rs2023239| rs12720071| rs2180619): positive associations were found at the 2-4 SNPs levels for 7 haplotypes reported in Table 30.

Interestingly, CNR1 variations (rs1049353 - rs2023239 = 0.00) were already found significantly associated with marijuana-related problems among regular marijuana users; the combination of higher trait impulsivity and CNR1 variation was associated with a greater number of marijuana-related problems (Bidwell et al, 2013).

Environmental factors

The influence of environmental factors on the risk of marijuana addiction was assessed by three psychometric tests (ACES, CECA-q, PBI) in Caucasian population. Logistic regression models revealed 4 parameters, gender, parental bonding, emotional neglect and physical neglect, as preconditions to cannabis addiction development. Subjects who declared *emotional* and *physical neglect* shows a risk respectively about 11.5 and 8 times higher to develop cannabis use disorders than subjects who do not have the perception of these psychological and physical damages. In addition, subjects who declare an optimal parenting has approximately a risk 60-70% lower to be marijuana users. When the significant variables coming out from the first model were included in a second model together with the genotypes, none of the genetic variants showed significant relation with marijuana use, however the psychometric variables *emotional neglect*, *physical neglect* seem to be confirmed as risk factors to cannabis use disorder.
In the American population, it was not possible to perform the psychometric analyses instead an environmental evaluation was performed considering the environmental risk factors from the demographic data collected for each subject: the four environmental risk factors considered are *gender, marital status, level of education, BMI*. Among these factors, an increase in education by one level, drops the risk of marijuana use by approximately half.
In both the populations the gender difference affected cannabis use disorder development, male present a higher risk to develop cannabis use disorders, compared to female, underlying the importance to investigate gender difference in this area of research. The result is consistent with other studies where gender differences in cannabis use often emerge, with males tending to use more frequently or at a higher rate than females (Agrawal & Lynskey, 2007; Perkonigg et al., 2008; Farmer et al., 2015).
Observations on environmental data suggest the existence of distinguishable types of trajectory for development of cannabis use disorders and emphasize the need to increase environmental evaluation and above all environmental risk factors perception, especially during the last steps of childhood and among adolescents. Neglect is defined as the failure, refusal or inability on the part of a caregiver, to provide necessary care, to meet a child’s basic physical and/or psychological needs; the perception of this damage, the parental bonding features, represent silent damage which should not be underestimated. Take part of educational environment, as school, can be represent protective factors which offers a strong motivational alternative to refuse cannabis use.
Other epidemiological research aimed to identify predictor factors for cannabis initiation and cannabis use disorders. Early life events play a role in trigger cannabis use during adulthood and this association may be due to exposure to stressful life events during youth and high levels of stress during early adulthood (Perkonigg et al., 2008). Adolescent cannabis use was already associated to the experience of stress attributed to familial instability, and familial disruption through divorce or death, for example, was hypothesized to contribute to the initial choice to use cannabis (Flewelling & Bauman, 1990; Butters, 2002). An evaluation of psychosocial risk and protective factors on cannabis use disorders showed increased risk of having cannabis use disorder consisted of higher number of deviant and susceptibility to peer drug use, skipping work more often and more frequent violence (Brooks et al, 2011).

Early childhood maltreatment was studied to investigate how this damage may influence cannabis use and abuse via personality and psychopathology; the study provided a developmental sequence (Figure 58) where childhood maltreatment severity affects less adaptive childhood personality functioning, followed by externalizing problems in preadolescence, and adolescent cannabis addiction symptoms (Oshri et al., 2011).

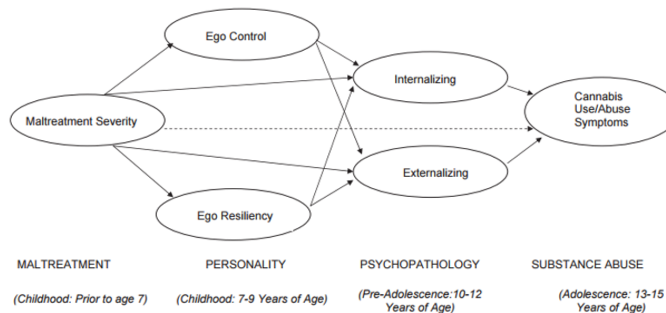


Figure 58. Developmental sequence of environmental factors for cannabis use and abuse symptoms (Oshrie et al., 2011).

These observations prompt to consider that environmental factors may represent more than a simple association or a consequence of cannabis use disorder, but the factors that mainly contribute to this risk. These findings suggest furthermore the importance to provide a protective environment, especially family and school or educational environments in general, and to exploit their role as potential prevention strategies and intervention target. Broader social programs and policies in this context might be a relative easy accessible path for prevention during late childhood or early adolescence and to promote program of special assistance to children who are at risk.

Epigenetic factors

Epigenetics may provide a biological mechanism for environmental exposure to adapt human beings in different situations. The interaction between genotype and environmental factors can be mediated by epigenetics mechanisms that can influence drugs of abuse response and dependence (Nielsen et al., 2012). This thesis work aimed to highlight DNA methylation, an epigenetic mark that generally leads to transcriptional silencing of the corresponding gene (Jones, 2012) and differences between marijuana users and control subjects. DNA methylation status was found significantly higher in marijuana users compared to control subjects in two of the genes analyzed: (1) hypermethylation at the exon 8 (+66.7 kb from TSS) within the DRD2 gene body ($p<0.034$) and (2) hypermethylation at the CpG-rich region (+3 kb from TSS) within the NCAM1 gene body ($p <0.0004$).

DRD2 is a vital part of the dopaminergic system (Ma et al., 2014). Alterations in DNA-methylation of the DRD2 gene have been associated to abstinence from pathologic gambling. Patients without gambling behavior showed lower DRD2-promoter methylation and participants with gambling behavior showed highest methylation levels (Hillemacher et al., 2015).

Because DNA-hypermethylation in most genes is usually associated with transcriptional repression and lower mRNA expression, they assumed the subsequently higher availability of D2 receptor results in a more efficient working reward system. According to our results, we could hypothesize (Figure 59) that increased DNA methylation observed in marijuana users might result in a lower D2 mRNA expression, lower availability of the receptor and an inefficient subsequent working reward system. This impaired function in the reward system, supported by other genetic and epigenetic alterations, may lead to less necessity for stimulation in non-marijuana users, and a higher necessity of stimulation, carried out with cannabis use, in marijuana dependent subjects.

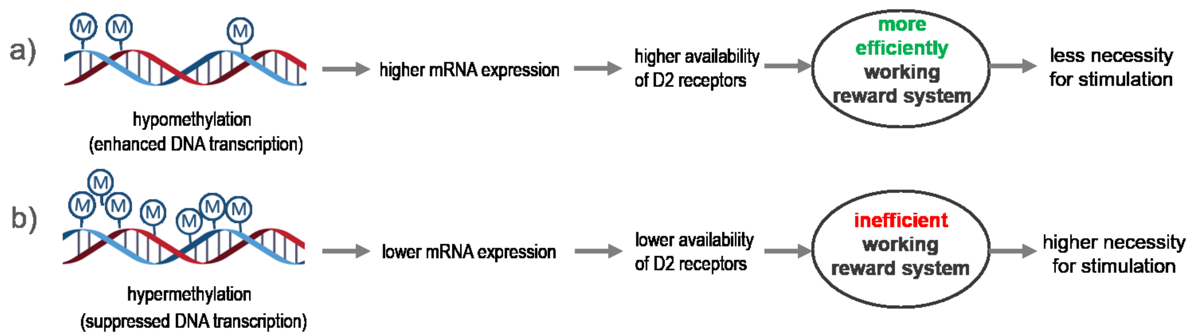


Figure 59. (a) Hillemacher's (2015) hypothesis on altered DNA-methylation in pathological gambling patients (b) Based on the current results, hypothesis of DNA hypermethylation in patient with cannabis use disorders.

Recent studies have investigated the relation between the D2 receptor promoter methylation, bulimia-spectrum disorder and environmental factors, as prior experiences of childhood abuse and comorbid Borderline Personality Disorder (BPD).
DNA hypermethylation on the promoter region of DRD2 was observed in women with a BPD, compared to women with no eating disorder, and marginally increased DRD2 methylation when compared to women with a bulimia-spectrum disorder but no comorbid BPD (Groleau et al., 2014). These results were reported to be consistent with findings that associate low-dopamine function (due to increased DNA methylation) and traits of emotional dysregulation (Eisemberg et al., 2007). Even more interestingly they noted the DNA methylation was higher in bulimia-spectrum disorder women who also reported experiences of childhood sexual abuse, compared to the control group, but not compared to women with a bulimia-spectrum disorder and no childhood sexual abuse. This observation suggests, in line with a growing body of literature, stressful life events as childhood maltreatment experiences may lead to epigenetic changes that impact gene expression (Perroud et al., 2011; Groleau et al., 2014).
In addition, these environmental negative episodes could alter the dopaminergic functioning and act as the trigger, later in life, for these multifactorial conditions, as addiction or psychiatric disorder. In support of this hypothesis, the DNA methylation frequency was also found different in the promoter of DRD4, DRD5, and DRD2 genes between blood samples of schizophrenia patients and healthy controls. The alteration reflected also mRNA expression level changes between schizophrenia patients and controls (Kordi-Tamandani et al., 2013).
It is important to notify that the analysed region in DRD2 gene (+66.7 kb from TSS, exon 8) is located close to the SNP Taq1A, rs1800497, of ANKK1 gene. Van der Knaap and colleagues (2014), in a trial study, explored weather the association between COMT gene methylation, and substance use was dependent on the COMT Val 108/158 Met polymorphism in human blood samples. COMT enzyme acts in the dopamine degradation pathway. Adolescents with the Met/Met genotype of COMT gene in combination with high rates of COMT promoter methylation is less likely to be high-frequent cannabis users when compared to adolescents with the Val/Val or Val/Met genotype (van der Knaap et al., 2014). It was therefore interesting to explore if the association between DRD2 gene methylation and marijuana use was dependent on the ANKK1 Taq1A polymorphism in the American population. For this purpose, Chip primers for DNA methylation analysis in the exon 8 of DRD2 gene at +66.7 kb from the transcription start site (TSS) were projected. No significant differences between marijuana users and controls resulted from genotyping analysis, but the DNA methylation was significantly higher in marijuana users compared to control subjects.

It could be assumed that genetic factors alone could play a restricted role by themselves, but environmental conditions and stressful life events, through epigenetic modifications, might have a key role triggering addiction development. It is evident that a complex relationship exists between genetic and epigenetic interactions, and even more complex the interplay between peripheral epigenetic marks and methylation status (Szutorisza and Hurd, 2016).

Increased DNA methylation at the CpG site within NCAM1 gene body (+3 kb from the TSS) was found in marijuana users compared to controls. NCAM1 is involved in a wide range of brain functions, including neuronal adhesion, neurite outgrowth, synaptic plasticity, and signaling transduction (Walmod et al., 2004).

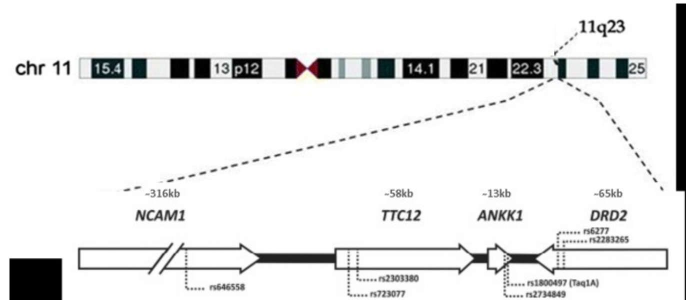


Figure 60. The NTAD cluster in the human genome, showing the single nucleotide polymorphisms (SNPs) studied by Mota (2015). Only DRD2 is positioned in the reverse strand (arrow pointing to the left) (Mota et al., 2012).

A recent GWA study found four significant genes associated with lifetime cannabis use: one, of these four genes, is NCAM1 that is part of the NCAM1–TTC12–ANKK1–DRD2 gene cluster (NTAD) (Figure 60). NTAD cluster is related to neurogenesis and dopaminergic neurotransmission and it has been associated to nicotine dependence (Gelernter et al., 2006) and hypothesized to be associated with other substance use disorder (Ma et al., 2014; Stringer et al., 2016). Mota and coworkers (2012) analyzed NTAD cluster with the other 46 available vertebrate genome sequences in BLAST searches and it resulted in conserved synteny (genes in the same chromosome) and neighborhood (genes side-by-side in the same order) of the whole NTAD cluster for 46% of the sequences available. Since the natural selection has maintained the NTAD cluster practically intact for at least 400 million years, they assume adaptive functional properties for NTAD cluster: variants in these genes with essential functions in neurogenesis and dopaminergic neurotransmission had probably played a role in the emergence of an efficient of adaptive advantage and for establish novel behavioral traits (Mota et al., 2012). Cell adhesion molecules of the immunoglobulin superfamily have been shown to play an important role in structural re-organization and signal transduction mechanisms in assessing learning and memory (Weltz et al., 2003); stress can also affect cell adhesion molecules expression (Sandi, 2004), resulting from elevation of glucocorticoid levels and altered glucocorticoid receptor expression, and cognitive functions through structural modifications of nervous system connections (Oitzl et al., 2000). Stress early in life causes delayed impairments of brain functions (Kosten et al., 2008) and silencing of adhesion molecules genes via DNA methylation could be the responsible mechanism (Desarnaud et al., 2008).

Moreover, rodent studies proposed NCAM as a modulator of the dopaminergic system and a potential pharmacological target for dopamine-related psychiatric disorders (Mota et al., 2012), because NCAM can regulate DRD2 signaling by promoting DRD2 internalization/desensitization and subsequent degradation (Xiao et al., 2009).

DNA methylation profile appeared to be associated with alcohol dependence in a population-specific way and the predisposition to alcohol dependence may result from a complex interplay of genetic variation and epigenetic modifications: CpG regions of genes, including NCAM1 and DRD4, were hypermethylated in alcohol dependence cases compared with controls (Zahng et al., 2013). Since these DNA methylation

changes observed in peripheral blood reflected effects on brain tissues, they were suggested as biomarkers for the prevention and treatment of alcohol dependence (Barker et al., 2013).

Recently, ANKK1 mRNA and protein has been shown to vary along neurodevelopment in the human and mouse brain and during the cell cycle in neural precursors (España-Serrano et al., 2016). Taken together these observations might support a role for the NTAD genes and TaqIA-associated phenotypes to brain structure development.

In this study, we found significant increased DNA methylation in two genes of this cluster (NCAM1 and DRD2) in marijuana users compared to control subjects, denoting the importance of approaching the NTAD cluster as a candidate functional unit, rather than its genes separately, in behavioral and psychiatric genetic studies. Identify epigenetic regulatory mechanism on this gene might help to understand the complex interplay between environment and human behavior and could open new windows for the development of therapeutic drugs in drug dependence and psychiatric disorders.

6. Conclusion and Future perspectives

The goal of this project was to investigate the genetic, environmental and epigenetic factors that may influence marijuana use disorders. In recent years, researchers have shed light on Taq1A polymorphism of ANKK1 gene and addiction. This study confirmed a correlation between this genetic polymorphism and the risk of marijuana use. In addition, epigenetic factors, precisely DNA methylation in specific region of DRD2 and NCAM1, have been revealed, for one of the first times, to interact with marijuana use. Epigenetic factors may contribute to the susceptibility to this multifactorial condition and/or vary the individual response to the drug or could be modify by marijuana itself during life. All these genetic and epigenetic factors belong to dopaminergic pathway, emphasizing its role in addiction development, even in marijuana addiction, despite misconception that it is not addictive and less harmless compared to other substances.

Few points should be considered. An interesting, environmental factors as education and the perception of parental care were revealed important contributor of marijuana use and perhaps the most direct route to prevent marijuana use disorder later in life. Different factors as drugs, social/behavioral, diet, stress have all been shown to be associated with human environmental exposures through epigenetic changes. It is important to remind that biomarkers research is still in a first stage of discover; DNA methylation modification, among all the epigenetic modifications, can last for many years, shows specificity and is present in accessible tissues. It is therefore suitable features to serve as a robust exposure biomarker, given that chronic effects of exposure on DNA methylation may be present after many years (Ladd-Acosta et al., 2015). It is furthermore important to remind that a case-control design have been used to analyze DNA methylation in marijuana addiction. Unlike the genome, DNA methylation is sensitive to both genetic and environmental factors, raising difficulties in establishing causal pathways (Cecil et al., 2016): the difficult to understand if the source of epigenetic changes comes from drug exposure or from trauma and stressful conditions still remains. It is, thus, problematic to establish whether identified higher DNA methylation level are a predisposing factor for marijuana use and/or a consequence of long-term use.

As previously noted (D'Addario et al., 2013), these findings suggest epigenetic regulation in peripheral blood lymphocytes are confirmed as an easily accessible biological marker for the study of THC action and dependence.

The present study has certain limitations. First, the sample size could be increased for genotyping analysis to obtain precise results. Second, it was not possible to maintain the same workflow in both Caucasian and American populations since the samples were collected in two different geographical location. Performing all the analyses in a structured project, with homogeneous variables, might give more reliable and exhaustive results. For example, evaluation of similar psychometric analyses and epigenetic analyses could have added more. mRNA levels measurement and protein assays in addition, may lead to verify whether observed methylation alterations in gene affect gene transcription and protein translation.

Third, DNA methylation level measured in blood cells may not reflect those in brain tissues. As the rewarding effect of marijuana is mediated by the brain's reward center, it would be more appropriate to use human postmortem brain tissues to study DNA methylation in relation to marijuana use. Unfortunately, human brain tissue samples are not easily accessible. Mouse brain tissues could be used for a replication study, comparing blood and brain, and clarify differences and equality found among control and addictive subjects. Another limitation is that DNA methylation has typically been examined at a single time point, precluding the possibility of examining how marijuana exposure and DNA methylation change over time influences addiction risk. Finally, given the fact that marijuana use disorders is a complex multifactorial condition, it is likely that genetic and epigenetic variations in many genes contribute to develop the risk of addiction: some functionally important CpG sites or other gene loci may have been missed; therefore, a

high-density methylation study across the genome and genomic array analyses could better examine the association of risk polymorphisms, DNA methylation and marijuana use.

In conclusion, this study is one of the first to investigate the association between environmental factors, genetic polymorphisms, DNA methylation and marijuana use in two different populations. Further exploration of the obtained results from this study would be important to understand if genetic polymorphisms, stressful life events and differences in DNA methylation of marijuana-associated genes could serve as biomarkers for the prevention and treatment of marijuana use disorders.

APPENDIX

A1. GENETIC POLYMORPHISMS ANALYSED AND DRUG ADDICTION ASSOCIATION

Table 1a. The list of candidate genes, the analysed polymorphisms and relative associations with references are reported.

Gene	SNP/VNTR	DNA sequence variation	Position	Functional Conseq.	Association	References
FAAH	rs324420	A/C (FWD)	1:46405089	missense: Pro ⇒ Thr	AA genotype higher propensity to addiction	Sipe et al., 2002
					AA carriers more likely to try cannabis	Tyndale et al., 2007 Kathuria et al., 2003
					Associated with cannabis use disorder in association with rs4141964	Melroy-Greif et al., 2016
CNR1	rs1049353	A/G (REV)	6:88143916	syn codon: Thr ⇒ Thr	AA carriers - high impulsivity when exposed to psychosocial adversity	Buchmann et al., 2015
					alcohol dependence	Marcos et al. 2012
					Risk factor for the development of cannabis dependence, in association with rs806380	Hartman et al., 2009
	rs2180619	A/G (FWD)	6:88168233	upstream variant 2KB	GG genotype more frequent in subjects with substanceuse disorders	Zhang et al. 2004
					GG subjects higher risk for high anxiety	Lazary et al. 2009
					GG subjects, general lower performance in the task problems in working memory and higher vulnerability to distractors	Ruiz-Contreras et al., 2014
	rs806379	A/T (FWD)	6:88151548	intron variant,upstream variant 2KB	AA carriers present enhanced impulsivity in early adversity conditions, in association with the rs1049353 T allele	Buchmann et al., 2015
					T allele or T-positive genotypes show a rapid onset of psychosis after methamphetamine abuse	Okahisa et al., 2011
	rs6454674	G/T (FWD)	6:88163211	intron variant	increase BMI	Benzinou et al., 2008
					rs6454674-rs806380-rs806377-rs1049353 (GGCC, TACC, GACC) associated with cannabis problems	Hopfer et al., 2007
					Significant associated to drug and alcohol dependence	Zuo et al., 2007
	rs12720071	A/G (REV)	6:88141462	utr variant 3 prime	Nicotine dependence, haplotype study	Chen et al., 2008
					Interaction effects genotype and marijuana use on white matter volumes and neurocognitive impairment	Ho et al., 2011
	rs2023239	C/T (FWD)	6:88150763	intron, upstream variant 2KB	more severe form of depression in AA carriers	Mitjans et al., 2012

					Haplotype rs2023239-rs12720071-rs806368 was associated with nicotine dependence	Chen et al., 2008
CNR2	rs2501431	A/G (FWD)	1:23875153	syn codon: Gly⇒ Gly	Nominally associated with response in a subset with fear-based anxiety disorder diagnoses	Lester et al., 2016
COMT	rs4680	A/G (FWD)	22:19963748	Missense upstream variant 2KB: Val ⇒ Met	Altered enzymatic activity	Männistö & Kaakkola, 1999; Chen et al., 2004
					Increase the vulnerability to cannabis dependence	Baransel et al., 2008
					Val allele carriers most sensitive to THC-induced psychotic experiences	Henquet et al., 2006
					Val allele carriers associated with psychotic symptoms	Caspi et al. 2005
					Met allele carriers associated with cannabis use in schizophrenic patients	Costas et a., 2011
					interaction with cannabis use in people with an At Risk Mental State (ARMS)	Nieman et al, 2016
DRD2	rs6277	C/T (REV)*	11:113412737	syn codon: Pro ⇒ Pro	Affect gene expression, modifying mRNA stability in combination with G1101A mutation	Duan et al., 2003
					Changing DRD2 density in cortex and thalamus	Monakhov et al., 2008
					Decisions associated with negative reinforcement outcomes	Jutras-Aswad et al., 2012
					CC genotype overrepresented in alcohol-dependent patients with dissocial personality disorder in association with TaqI-A SNP	Ponce et al., 2008
					Low value measuring reward sensitivity	Davis et al., 2008
					C/C genotype strongly associated with schizophrenia	Lawford et al., 2005
ANKK1	rs1800497	C/T (REV)	11:113400106	missense: Glu⇒ Lys	Association with alcohol and drug dependence	Yang et al., 2008
					Low dopamine receptor density	Jönsson et al., 1999
					Lower binding potential of D2R	Gluskin et al., 2016
					Higher risk of alcohol use, in association with conduct disorder or impulsive behavior	Esposito-Smythers et al., 2009
					Increased risk of cannabinoid dependence	Nacak et al., 2012
DAT1	VNTR 3'UTR	40bp, 3-11 repeats	5p15.3; 3'UTR	3'UTR	altering mRNA structure and consequently DAT expression levels	Fuke et al., 2001
					9R carriers have higher striatal DAT availability and higher striatal DAT expression	van Dyck et al., 2005; van de Giessen et al., 2009

					Risk factor for cannabis dependence in association with rs806380	Hartman et al., 2009
					9R/9R genotype confers general protective effect against risky behaviors, including marijuana use	Guo et al, 2010
DRD4	VNTR exon 3	48bp, 2-11 repeats	11p15.5; exon3	exon 3	7R+ may intensify risk for problematic tobacco and cannabis use	Olsson et al., 2013
					In a sample of primarily cannabis users DRD4L was linked to problematic illicit drug use	Kendler et al., 2008
					DRD4L homozygotes greater frequency of Marijuana	Mallard et al., 2016
					DRD4 7-repeat cannabis use related to parental monitoring	Otten et al., 2013

A2. THE NEUROBEHAVIOR OF DRUG INTAKE IN RODENT SELF-ADMINISTRATION MODEL

From February to November (2016) I have been as Special Volunteer at National Institute on Drug Abuse (NIDA). As my interest was to gain further knowledge in latest techniques in the field of neuroscience, I have pursued to learn advanced surgical experiments in rat model to study the neurobehavior of drug intake. Here the rats receive a catheter implantation for intravenous self-administration of drug. Administration of various doses of drugs of abuse can alter gene expression and these alterations may depend on drug-induced epigenetic changes, including histone acetylation and/or DNA methylation/demethylation. Specifically, I had the possibility to participate at two different projects in the Molecular Neuropsychiatry Research Branch (MNRB) of the National Institute on Drug Abuse (NIDA), Baltimore, MD, USA: the first one was to test if a dopamine D1-like receptors antagonist was able to reduce the Methamphetamine (METH) intake in rat self-administration model. The second project was to analyse DNA methylation, mRNA expression and protein expression of potassium channels in brain samples from rats previously trained with prior injection of methamphetamine followed by METH self-administration. DNA methylation was performed through MeDIP-qPCR, mRNA and protein expression was performed through respectively Real Time PCR and immunoprecipitation followed by Western Blot with advanced Chemiluminescent techniques. Following these experiments, a potassium channel blocker in rat METH self-administration model was tested.

Self-administration

In self-administration studies the experimental timeline consists of five main phases: a habituation period of the animals, surgery step followed by recovery, the self-administration training phase that allows to simulate the addictive behaviour, a withdrawal phase with extinction testing and biochemical and molecular analyses of the various brain regions collected (Figure 1a). The time indicated for each step may vary as per the aim of specific experiment.

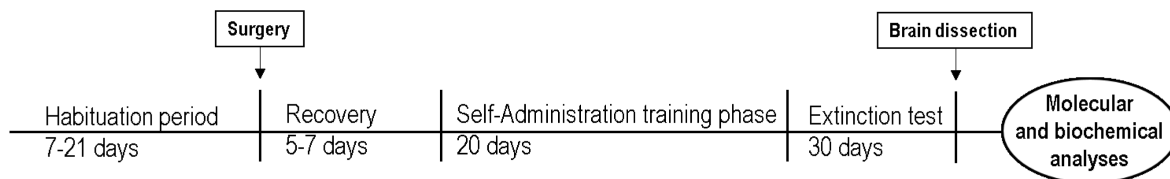


Figure 1a. The self-administration experimental timeline.

The experiment started with a first habituation period prior to surgery of 7-21 days (rats were maintained on a reverse light-dark cycle during the entire experiment). The surgery consisted of a catheter implantation into jugular vein and a back-mount procedure for delivery of intravenous self-administration of the drug. After surgery, rats were maintained and monitored in the animal facility for 5-7 days. During this period, the rats were handled daily and the catheters were flushed every day with sterile saline containing Gentamicin (5 mg/ml, 0.05 ml). After recovery, the rats were moved to the operant chambers where they lived during the training phase (Figure 2a) of the experiments (approximatively 20 days).

A subgroup of the rats also had access to self-administer saline, instead of self-administration of the drug. This saline group represented the control group, to compare, subsequently, the data and samples from the rats that received the drug.
Food pellets and water were continuously available in the self-administration chambers and the rats' body weights were monitored every day and recorded in a notebook. Each operant box had two levers located 9 cm above the floor, but only one lever (an "active", retractable lever) activated the infusion pump that administered the drug through the jugular vein (responses on the inactive lever were recorded as control). Each drug infusion is paired with a 5 second tone-light compound cue. For 20 secs after the drug infusions, a timeout period prevented drug overdose and, in addition, the number of drug infusions per day was limited. Rats were trained in 3x3 or 3x2-hour sessions, separated by 30 min.
The self-administration sessions started at the onset of the dark cycle and sessions began with the insertion of the active lever and the illumination of a red house-light that remained on for the duration of the session. At the end of each 3-h session, the house light was turned off, and the active lever was retracted.

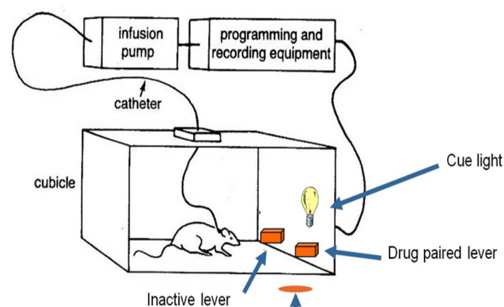


Figure 2a. The self-administration chambers.

The aim of these experiments was to achieve a reduction in drug self-administration in rats. To this purpose, during the self-administration sessions different options could be added to this schematic experimental timeline (see below "Foot-shocks role in the self-administration rat model" and "Pharmacological drug treatment paragraphs").
Animals were then allowed to withdraw from the drug for a particular time period. This is called the extinction phase, where testing is done in the same environmental conditions as the self-administration training. Rats are brought back from the standard cage housing but now will no longer receive intravenous drug injections or foot shocks that were given previously. Total number of lever presses are recorded during this session. This was because it has been shown that cue-induced drug seeking increases over the withdrawal period, a process called incubation of drug craving. The number of lever pressing is considered as a measure of how much animals wanted the drug.
The behavioral experiments are completed following the last extinction test session. The rats were returned to their home cages and subsequently brain dissection was carried out to collect regional tissues for molecular and biochemical analyses.

Foot-shocks role in the self-administration rat model

To achieve a reduction in drug self-administration in rats an approach to changes the consequences of drug intake from positive to negative was used. It is important to note that many people start to use drug, but only a low percentage of them develop addiction. In addition, human substance users reduce or stop their drug use when they face up with negative consequences. In contrast, individuals who become addicts continue to use the drug despite the adverse consequences associated with the continuous drug abuse (Cadet et al., 2016). Rat self-administration models are an appropriate tool to study potential molecular bases of drug taking behaviors by humans (O'Connor et al., 2011), but it is difficult to distinguish in the rat model, humans who reduce or stop their drug use because of the negative life events caused by drug use, from the real addictive one. Cadet and co-workers, in a recent study, to simulate the adverse consequences of drug intake, added mild foot-shocks, during self-administration

training, to divide rats into animals that continue to press a lever to get the drug (shock-resistant) and those that significantly reduce pressing the lever (shock-sensitive) despite the shocks (Cadet et al., 2016). In this kind of experiment the chamber was thus equipped with a grid floor that was connected to a initially low intensity intermittent foot-shock that was increased over time to punish the drug seeking response.

Pharmacological drug treatment

During the self-administration training phase to achieve a reduction in drug self-administration in rats, it was also possible to administer a pharmacological treatment via subcutaneous injection, depending on the pharmacokinetic properties of the administered drugs, based on literature research.
Based on which kind of pharmacological drug were used, this allows to assess gene and protein expression resulting from drug treatments and to investigate the role of specific receptors in maintaining compulsive drug self-administration.

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