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Pharmacogenomic analysis of Neuroleptic Malignant Syndrome

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ABSTRACT

Neuroleptic Malignant Syndrome (NMS) is a rare but potentially fatal adverse drug reaction (ADR) to the administration of drugs acting on the central dopaminergic system, such as first-generation antipsychotics, namely "typical" or FGA, and second-generation antipsychotics, namely "atypical" or SGA. Cases of NMS triggered by the assumption of antidepressants, lithium, certain antiemetics, illicit substances of abuse (amphetamine or cocaine) or sudden withdrawal of dopaminergic medications have also been reported. Severe and milder cases of NMS have been described. Main clinical features of NMS include hyperthermia, muscle stiffness, mental status alterations, creatine kinase elevation, blood pressure instability and heart rate increase. Since other disorders shares clinical sign and symptoms with NMS, the diagnosis is based on exclusion criteria. According to the 5th edition of the *Diagnostic and Statistical Manual of Mental Disorders*, exposure to antipsychotic triggers and exclusion of other etiologies are key points for an accurate NMS diagnosis.

The onset of NMS is not preventable. High ambient temperature, dehydration, concomitant medical conditions, polypharmacy, initiation of pharmacological treatment or drug dosage modifications have been recognized as risk factors that may contribute to develop NMS. Depending on antipsychotics (APs) and healthcare treatments, about 0,02-3% patients may develop NMS, of which about 10-30% has a fatal outcome. Although the precise pathophysiology underlying NMS remains unknown, a reduction of central dopaminergic activity in the central nervous system due to blockade of D_2 receptors is compatible with the typical clinical features of the syndrome and it may play a key role to trigger NMS.

Genetic variants of genes encoding the receptors of dopamine and serotonin and cytochrome P450 2D6 have been studied in association with NMS susceptibility, mostly in the Japanese population, with conflicting results so far. Thus, no genetic biomarkers for NMS predisposition have been clearly identified yet.

In the current study, a joint collaboration among the Poison Control Centre (PCC) and National Toxicology Information Centre (CNIT) of the IRCCS ICS Maugeri, Pavia, Department of Biology and Biotechnology "L. Spallanzani", University of Pavia and Institute of Molecular Genetics "L. L. Cavalli Sforza" has been activated to investigate a cohort of well-characterized south European non-Finnish patients who developed NMS upon antipsychotic therapy.

Initially, allelic variants of the dopamine D_2 receptor gene (*DRD2*) were studied on a total of 17 AP-triggered NMS patients to compare these results with the first conflicting data obtained from the Japanese cohort. Subsequently, whole genome sequencing (WGS) of 11 NMS patients from the European cohort was performed to identify copy number variations (CNVs) and structural variants (SVs) shared among these NMS patients in order to identify a genetic trait.

Restriction fragment length polymorphism analysis on the GG to G- variant (rs1799732) located in the promoter region of the *DRD2* revealed that 7 of 17 NMS patients carried the rare G-allele, of whom 6 patients developed NMS upon SGA monotherapy. Thus, the G- allelic frequency raised from f=0.071 among healthy individuals (9 subjects out of 70) to f=0.21 of NMS patients. The G- allelic frequency of control cohort (f=0.071) was slightly lower compared to the allelic frequency reported in GnomAD, thus the G- allele frequency raised about 2.2-fold from about 0.097 of healthy European non-Finnish population to 0.21 of the NMS patients. By applying the dominant model analysis, the presence of at least one G allele (G/GG heterozygote or G/G homozygote, namely G-carrier) showed statistically significance increase of 3.2-fold ($p<0.05$) of G-carrier among NMS patients (f=0.412) compared with healthy control individuals (f=0.129). Thus, the susceptibility to develop NMS in G-carrier patients increased about 5.5-fold compared with the G-non-carrier patients (GG/GG) following antipsychotic therapy.

These results highlight a risk factor to be evaluated before treating patients with an AP therapy if it based on SGA treatment. These results suggested that this SNP mapped in the *DRD2* promoter may impair or change the DNA binding affinity of transcription factors controlling this gene transcription.

To deeply investigate whether genetic predisposition and/or genomic traits could be associated with NMS susceptibility, whole genome sequencing (WGS) was applied on a subset of patients who developed NMS. The gDNAs of these patients were sequenced at 30x coverage and quality control revealed a phred_20 and a phred_30 score above 96% and 91%, respectively. More than 98% segments were aligned to the GRCh38 reference genome at 10X coverage and more than 45% at coverage 30x. All these parameters indicated excellent quality of WGA.

To summarize, almost 900 CNVs (copy number variants) of which about 700 gain and 200 loss, more than 10,000 SVs (structural variants) and more than 5 million SNPs/INSDELs were discovered on average for each patient. With the purpose of discover CNVs and SVs shared among all the NMS patients, the WGS data were intercrossed. Seven shared CNVs were found, two of which were not further investigated since commonly reported among European population. Of the remaining 5 CNVs, three losses (chr13q34, chr17 and chr21p13, respectively) were mapped on chromosome bands or nearby the centromeric region in which no genes or structural variants were annotated yet. The fourth identified CNV loss of 110 kb was mapped on chr16. This CNV was never observed among healthy European individuals, in this region two pseudogenes and 3 new lncRNAs with unknown function were annotated. The last identified CNVwas a 3 copy gain of 170kb length mapped at chr17. It was never described and no CNV frequency is available. A gene encoding an inward rectifier potassium channel (*KCNJ18*) has been mapped in this genomic interval. Hence, a 3-copy number increase of the *KCNJ18* gene could raise the inward potassium flow by the potassium channel Kir, with higher potassium concentration. Thus, a potential hyperkalemia could be related to muscle damage and rhabdomyolysis, known as two of the major clinical signs of NMS.

Moreover, among the 10,000 structural variants found on average per patient, about 1024 SVs (10%) were shared among all patients, of which 435 occurred in protein coding regions. Tissue expression tools were applied to select genes expressed in the brain or involved in the pharmacokinetics and pharmacodynamics of antipsychotics. Two SVs (an insertion in heterozygosity and a 327 bp deletion in homozygosity) were identified affecting the adrenoreceptor alpha 1B (*ADRA1B*) and the dopamine D3 receptor (*DRD3*) genes respectively, both widely expressed in the CNS and main target of SGAs.

The *ADRA1B* adrenoceptors act as stimulatory receptors involved in vascular muscle and in local vasoconstriction, as well as blood pressure and temperature control, signal transduction and calcium signalling pathways. Therefore, genetic alterations of *ADRA1B* could cause dysregulation of the signal transduction cascade controlling blood pressure and body temperature, whose clinical signs have been described in NMS patients. Moreover, the 327 bp deletion mapped in the promoter region of *DRD3* likely affects the expression of this gene encoding a dopamine receptor mainly expressed in the limbic system and associated with regulation of cognitive and emotional functions, reward mechanisms and motor control. The lack of the D3 dopamine receptor in these brain regions could dramatically reduce dopamine binding capability, leading dopamine uptake near to a critical threshold that could trigger NMS if the patient requires a dopamine antagonist therapy with APs.

In conclusion, it seems that genomic variations involving dopamine D_2 and D_3 receptor genes are related to the early developmental stage of NMS, probably due to a downregulation or a blockade of the dopaminergic signaling cascade in the central nervous system, whereas genetic variations affecting the *ADRA1B* and *KCNJ18* genes would instead appear to explain the mechanisms involved in the typical clinical manifestation of NMS.

Thus, the genetic variants discovered in this study are candidate markers of NMS to be deeply investigated by functional assays, meanwhile these genetic markers should be evaluated in clinical practice for tailoring antipsychotic treatment with the goal of maximizing efficacy and reducing adverse drug reactions.

ABBREVIATIONS AND ACRONYMS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
AAD	Atypical antipsychotic drugs
ADHD	Attention-deficit hyperactivity disorder
ADR	Adverse drug reaction
ADRA1B	Adrenoceptor alpha 1B gene
ADRH	Antipsychotic drug-related heatstroke
ANKK1	Ankyrin repeat and kinase domain containing 1 gene
AP	Antipsychotic drug
BBB	Blood brain barrier
chr	Chromosome
CNS	Central nervous system
CNV	Copy Number Variation
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
CYP2C19	Cytochrome P450 family subfamily C member 19 gene
CYP2D6	Cytochrome P450 family 2 subfamily D member 6 gene
DA	Dopamine
DAT	Dopamine transporter
DGGE	Denaturing gradient electrophoresis
DIC	Disseminated intravascular coagulation
DRD2	Dopamine receptor D_2
DRD3	Dopamine receptor D_3
ds-DNA	Double Strand DNA
DSM-IV	Diagnostic and Statistical *Manual* of Mental Disorders- IV edition
DSM-V	Diagnostic and Statistical *Manual* of Mental Disorders- V edition
ECT	Electroconvulsive therapy
EM	Exstensive metabolizer
EPI	Epinephrine
EPS	Extrapyramidal symptoms
FGA	First-generation antipsychotic
GABA	Gamma-aminobutyric acid
GAD	Generalized anxiety disorder
gDNA	Genomic DNA
HGP	Human Genome Project
HVA	Homovanillic acid
IBZM	[^{123}I] Iodobenzamide
ID	Intellectual disability
IEC	International expert consensus
IM	Intermediate metabolizer

INDEL	Insertion deletion
kb	Kilobase
KCNJ18	Potassium Inwardly Rectifying Channel Subfamily J Member 18
lncRNA	Long-non-coding RNA
MAF	Minor allele frequency
Mb	Megabase
MC	Malignant catatonia
MDD	Major depressive disorder
MH	Malignant hyperthermia
MHPG	3-methoxy-4-hydroxy-phenylethyleneglycol
miRNA	Micro RNA
MPTP	Methylphenyltetrahydropyridine
NE	Norepinephrine
NFE	Non-Finnish European population
NGS	Next Generation Sequencing
NMS	Neuroleptic malignant syndrome
NMSIS	Neuroleptic Malignant Syndrome Information Service
NSAID	Non-steroidal anti-inflammatory drugs
PCC	Poison control center
PCR	Polymerase Chain Reaction
PD	Parkinson disease/ Parkinson’s disease
PET	Positron emission tomography
PM	Poor metabolizer
PTSD	Post-traumatic stress disorder
rCBF	Regional blood flow
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
SGA	Second-generation antipsychotic
SNP	Single nucleotide polymorphism
SNS	Sympathetic Nervous System
SNV	Single nucleotide variant
SPECT	Single-photon emission-computed tomography
SS	Serotonine syndrome
SSRI	Selective serotonin reuptake inhibitors
SV	Structural variant
TCA	Tricyclic antidepressant
TD	Tardive diskinesia
TD	Tourette’s syndrome
TPP	Thyrotoxic periodic paralysis
TSS	Transcription start site
UM	Ultrarapid metabolizer
VEP	Variant effect predictor
VMAT2	Vesicular monoamine transporter 2
WGS	Whole Genome Sequencing

1 INTRODUCTION

1.1 NEUROLEPTIC MALIGNANT SYNDROME

It was 1960 when, in France, Delay and his colleagues first described a pathological condition they called *"syndrome malin des neuroleptiques"* during early clinical trials involving antipsychotic drugs. A few years later, in 1968, the term was translated into English as Neuroleptic Malignant Syndrome (NMS) and has been called that ever since (Delay *et al.*, 1960).

Neuroleptic Malignant Syndrome is a rare but severe iatrogenic neurologic condition, which occurs mainly as an idiosyncratic reaction associated with the use of antipsychotic drugs (AP), dopamine-receptor antagonist (D_2) or the rapid withdrawal of dopaminergic medications (Langan *et al.*, 2012; Oruch *et al.*, 2017). This syndrome is characterized by distinctive clinical symptoms, such as hyperthermia, autonomic instability, muscle rigidity, mental status alteration, creatine kinase elevation, extrapyramidal syndrome (Gurrera *et al.*, 2011).

Fortunately, NMS has a low incidence, although its occurrence is typically unpredictable. Despite this, some risk factors such as high environmental temperature, malnutrition, dehydration, exhaustion, concurrent medical conditions, polypharmacy, initial treatment or dosage changes and also previous episodes have been identifies as possible triggers for the onset of this syndrome (Mann *et al.*, 2003; Pileggi and Cook, 2016). Moreover, the diagnosis of NMS can often be very difficult because several medical syndromes and disorders (neuropsychiatric, systemic, and drug-induced hypermetabolic disorders) shares symptoms with NMS (Velamoor, 2017).

Lately, increasing awareness of NMS by physicians and psychiatrists has led to the need to develop both standardized diagnostic criteria and recommendations for appropriate medical treatment, thus leading to a desirable decrease in morbidity and mortality due to this syndrome (Oruch *et al.*, 2017; Velamoor, 2017). Nevertheless, it remains necessary continuing further study on NMS, since the precise pathophysiology, the genetic predisposition and the correlation with other drug-induced hyperthermic disorders remain a yet quite unknown issue (Margetić and Aukst-Margetić, 2010; Velamoor, 2017).

1.2 PRELUDE AND HISTORICAL BACKGROWND

In the mid-50s of the last century the treatment of psychotic disorders, such as schizophrenia, improved significantly with the introduction of the first antipsychotic drugs, leading to an essential revolution in mental heal treatment (Mann *et al.*, 2003). Prior to the use of antipsychotics, psychiatric therapies relied on extremely radical somatic therapies: for example, convulsant therapy, initially used in the treatment of schizophrenia, as well as lobotomy, sedation, or even the extreme practice of sexual sterilization, all of which were used with the misguided aim of treating the mind by treating the body, often without any efficacy and causing inevitable and irreversible adverse effects to patients (Braslow, 1997; Mungo and Fornaro, 2007).

It is in this context that, in 1952, Jean Delay and Pierre Deniker discovered by chance that Chlorpromazine, an antihistamine derivative first used in 1951 by Henry Laborit to enhance the effectiveness of anesthesiologic techniques of controlled hibernation, had antipsychotic properties (Mungo and Fornaro, 2007). From that moment on, chlorpromazine became available on prescription, first in France and then its use in psychiatric practice spread throughout the world leading to the beginning of the pharmacological revolution in psychiatry (Braslow, 1997; Mungo and Fornaro, 2007).

Although antipsychotics do not cure schizophrenia, their use has revolutionized the treatment of this syndrome and other psychotic disorders, decreasing the disability and suffering associated with these disorders and allowing many patients to return to leading nearly normal and independent lives. Shortly after chlorpromazine came into clinical use, other antipsychotic drugs were rapidly developed and made available for clinical practice. However, it soon became clear that these drugs had serious undesirable side effects: among these, extrapyramidal symptoms (EPS) including parkinsonism, akathisia, dystonia, acute or tardive dyskinesia, described as an adverse effect of chlorpromazine and its derivate administration, and also of a new medication synthesized in 1958 at a Belgian laboratory by Paul Janssen, called haloperidol (Delay *et al.*, 1960; Mann *et al.*, 2003; Yen, Lung and Chong, 2004). Fortunately, most side effects are minor or not very serious. Some uncommon side effects, however, can result in permanent morbid sequelae or even lead to death (Mann *et al.*, 2003).

Among these neurological side effects due to antipsychotic medication, neuroleptic malignant syndrome remains one of the most considerable exception: although it had already been discovered and described in France during the first antipsychotic drugs' trials in the 1960s, it only began to be recognized in England by clinicians about two decades later, when the description of NMS

finally appeared in the Diagnostic and Statistical *Manual* of Mental Disorders, Third Edition (DSM-III) published by the American Psychiatric Association (Caroff, 1980; Mann *et al.*, 2003; Velamoor, 2017). Before these discoveries, however, patients with clinical manifestation similar to NMS triggered by phenothiazine (an organic thiazine compound) and not recognized as NMS were reported. Until then, the adverse drug reactions (ADR) due to neuroleptic were disregarded compared with their therapeutic effect on central nervous system (CNS) (Mann *et al.*, 2003).

During the following decade, NMS had been reported in France, England, Japan and even America, where some researchers began using the term neuroleptic malignant syndrome to describe similar cases, while around the 80's Caroff, using diagnostic criteria overlapping those indicated by Delay and Deniker, examined and described more than sixty cases of NMS (Delay and Deniker, 1968; Caroff, 1980; Mann *et al.*, 2003).

Once the dangerousness of this iatrogenic syndrome was recognized and accepted by the clinical community, over 300 cases of NMS began to be reported before 1986. Several scientific articles, studies and case reports began to be published in those years, initiating the need to develop standardized diagnostic criteria, and identifying therapeutic approaches and the best treatments to reduce as much as possible the morbidity and mortality of NMS patients (Delay and Deniker, 1968; Caroff, 1980; Mann *et al.*, 2003).

1.3 EPIDEMIOLOGY

1.3.1 Incidence

Between 1960 and the early 2000 it was estimated to have ranged from a high of 3.26% to a low of 0.01%-0.02%, with an average estimate of 0.99% overall calculated by analyzing epidemiological data available in the literature (J. R. Strawn, Keck and Caroff, 2007; Margetić and Aukst-Margetić, 2010; Tse *et al.*, 2015; Simon, Hashmi and Callahan, 2021).

Though rarely leading to death, it has been reported that 10-30% of NMS patients have a fatal outcome (Pelonero, Levenson and Pandurangi, 1998).

There are several retrospective studies that provide detailed variation in the incidence of the syndrome between years in different medical environments. In Japan, in a survey conducted between 1988 and 1990, 1,666 suspected cases of NMS were estimated, belonging to about two-thirds of Japanese structures. In the same years, Gelenberg and colleagues analyzed first 1,470 and then 1,450 patients in two successive years, finding only one case of NMS (0,07%) during the first year of observation (Mann *et al.*, 2003).

In contrast, Pope and colleagues reanalyzed the clinical records of several patients treated with antipsychotic medications in therapy at Belmont Psychiatric Hospital (USA) at different times: first they analyzed one year between 1984 and 1985, during which only 7 patients out of a total of 483 (corresponding to 1.4%) had developed certain or suspected NMS; next, they analyzed a 31-month period (between 1984 and 86), during which 13 patients out of 1162 (1.10%) developed the neuroleptic syndrome; lastly, Keck and his group analyzed the incidence of NMS at the same Hospital between 1986 and 1990 (amounting to 47 months) and reporting an approximately 7-fold decrease in cases (0.15%) (Pope, Keck and McElroy, 1986; Keck, Pope and McElroy, 1991; Tse *et al.*, 2015). These findings led Pope and Keck's group to conclude that NMS cases were likely decreasing over time due to improved awareness of early signs of the syndrome and increased pharmacovigilance by clinicians (Tse *et al.*, 2015).

In the early 1980s second-generation antipsychotic (SGA), also known as atypical antipsychotic drugs (AADs) or neuroleptics, began to be prescribed in order to reduce the unpleasant adverse effects induced by these medications. However, the incidence of NMS due to these categories of drugs is not well established (Caroff *et al.*, 2002). Investigations conducted in the mid-1990s by Sachdev *et al*. and by Williams and MacPherson, focusing on adverse effects

induced by clozapine, estimated an incidence of NMS due to this antipsychotic between 0.24% and 0.10%. These findings are analogous to the estimated incidence of NMS among typical antipsychotics treated patients (Mann *et al.*, 2003). In contrast, Kozaric-Kovacic *et al*. (1994) reported no cases of NMS among approximately 700 clozapine-treated patients, whereas they found 17 cases (0.59%) of NMS among 2,897 subjects treated with two different first-generation antipsychotic (Mann *et al.*, 2003).

We can state that the incidence of the syndrome is not homogeneous across all over the world, so it can only be considered as a rough estimate (Mann *et al.*, 2003).

1.3.2 Risk Factors

Despite NMS is frequently regarded in literature as an uncommon syndrome and its occurrence is considered unpredictable among antipsychotic medication users, a number of risk factors that could promote the onset of the syndrome have been evidenced. Among these, the risk factors that are mostly considered are: demographic risk factors (age and gender), environmental risk factors (like high ambient temperature, dehydration), pharmacological risk factors (type of medication, pharmacokinetics, polypharmacy) and genetic risk factors (Tse *et al.*, 2015).

Nevertheless, even NMS is not correlated with any specific psychiatric illness, some disorders associated with patients' general health conditions, including schizophrenia, mood disorders, brain disease and concomitant medical condition may play an important role in increasing the risk of developing NMS (Caroff and Mann, 1993; Mann *et al.*, 2003).

1.3.3 Demographic risk factors: Age

Potentially, NMS could arise in subjects of any age who have been exposed to an antipsychotic medication. Despite this premise, the syndrome has been found in many cases in young and middle-aged adults, whereas occurrences of this adverse reaction among young children or infants is quite rare. The most likely explanation for this trend could be that higher doses of antipsychotic drugs may be used for these individuals. Indeed, it has been estimated, that the average age of individuals who experience the syndrome for the first time is around 40 years (Mann *et al.*, 2003).

From a review conducted on the incidence of the syndrome among youths and children, Silva and colleagues found just under 80 cases of patients whose ages ranged from less than one year to

18 years. Among these patients, only 10 were younger than 11 years old and as age increased, so did the incidence of the syndrome. This trend probably corresponds to the lower rate of antipsychotics exposure in children (Silva *et al.*, 1999). In the early 2000s, Casteels-Van Daele and colleagues, reviewing the literature of Reye's syndrome cases in youngsters, highlighted the probability that primary cases of this disorder may actually have been unrecognized NMS cases occurred as an adverse effect of the antiemetic drug phenothiazines (Casteels-Van Daele *et al.*, 2000).

Until now, age has never been considered as a meaningful risk factor for the development of the syndrome (Mann *et al.*, 2003).

1.3.4 Demographic risk factors: Gender

As for age, gender is not a significant risk factor for the development of NMS. Several authors reported that NMS is more common among the male gender, other authors, otherwise, have reported that it is a more prevalent syndrome among the female gender; still other authors, finally, suggest that the syndrome is equally distributed between both genders.

In some studies, it has been reported that men have approximately 50% higher risk than women of being diagnosed with NMS. This observation could be mainly related to the physical characteristics of the male gender, namely the presence of greater muscle mass, which may consequently lead to greater muscle stiffness or support a higher metabolism leading to more pronounced hyperthermia and therefore triggering a more severe and recognizable development of the syndrome (Gurrera *et al.*, 2017).

According to some studies, it would appear that NMS is twice as common among male because antipsychotics are used differently depending on the gender of the patient. Indeed, it seems that men are more likely to be treated with high doses of antipsychotic medications, as they exhibit more positive symptoms, for example hostility and psychomotor agitation (Oneib and Zaimi, 2021). Therefore, disparities in antipsychotic drug exposure and dosages used to treat men and women have been suggested to explain the higher frequency of NMS in males, rather than factors related to inherent gender difference (Mann *et al.*, 2003; Gurrera *et al.*, 2017; Oruch *et al.*, 2017)
Environmental risk factors

Even if environmental factors do not play a primary role in causing the syndrome, they have been reported to increase the risk of NMS development. Environmental risk factors reported in the

global literature comprehend high external temperature, physical restraint, extreme agitation, exhaustion and dehydration due to insufficient fluid intake (Keck *et al.*, 1989; Mann *et al.*, 2003; Tse *et al.*, 2015).

Although some authors reported that cases of patients with NMS were more frequent in countries with warm temperate climates, NMS appears to occur at any ambient temperature and humidity condition, as cases of this syndrome have also been reported where the outdoor temperature registered as low as 8.5°C degrees (Mann *et al.*, 2003).

Furthermore, analyses of the occurrence of NMS showed that the syndrome did not have a higher incidence in warmer months (such as summer months in temperate climates) and that it occurred in all seasons. However, it would still appear that high temperatures and humidity may facilitate the onset of the syndrome as they would interfere with body heat dissipation mechanisms and therefore are consistent with the etiopathogenic pathways associated with the syndrome (Mann *et al.*, 2003; Tse *et al.*, 2015).

1.3.5 Pharmacological risk factors

Neuroleptic malignant syndrome is not correlated with any specific neuropsychiatric illness. In fact, there have been cases of patients who developed the syndrome as a result of taking antipsychotic drugs to treat various neuropsychiatric disorders, as well as NMS cases of patients without any psychiatric disorders who were treated with neuroleptic drugs used as sedatives or antiemetics (Mann *et al.*, 2003).

It has been reported that NMS can develop following administration of standard doses of the drug and also for all routes of administration. Despite this, it occurs more frequently during the beginning of treatment and/or for the initial months of therapy, or even after a change in therapeutic dosage, especially if it occurs suddenly. In this connection, administration of more elevated doses of antipsychotic medications have been correlated with an increased risk of developing NMS. Moreover, both intravenous or intramuscular routes of administration have also been correlated with an increased risk of NMS (Tse *et al.*, 2015).

Regarding the classes of medications, nearly all substances that can block dopamine D_2 receptors, including first- and second-generation antipsychotics and also certain antiemetics, have been associated with NMS. However, the high-potency first-generation antipsychotics (or "typical") appear to be correlated with a higher incidence of the syndrome compared to their

second-generation drug successors. The argument that could explain this hypothesis concerns the greater affinity of the first category of drugs, the FGAs indeed, to the dopamine D_2 receptors, which has a lower dissociation binding constant from the receptors although there is no evidence to support this appealing theory (Mann *et al.*, 2003; Tse *et al.*, 2015).

Second-generation antipsychotics (SGA, also known as "atypical" or "newer" neuroleptics) which have as their main feature a reduced affinity on the D_2 receptor and a higher affinity on serotonin receptors, seemed promising in reducing adverse effects with lower frequency than their predecessors. However, available data suggest that the incidence of NMS with second-generation antipsychotics is equal to or slightly lower than with typical antipsychotic medications, although these observations may be due to the fact that, recently, SGAs are prescribed more than first-generation antipsychotics. Contrary to what was initially thought, therefore, second-generation drugs are not exempt from the risk of triggering NMS (Pileggi and Cook, 2016).

Furthermore, some authors have suggested that NMS cases triggered by exposure to second-generation antipsychotic agents may sometimes occur with minor or milder symptoms, or even with significantly reduced signs of tremor and rigidity, making diagnosis more challenging. These milder cases, without all the major features of the syndrome, are commonly known as atypical NMS forms (Mann *et al.*, 2003; Belvederi Murri *et al.*, 2015; Uvais, 2017).

Although NMS can occur at any time during lifetime of patients treated with antipsychotics, pharmacological and treatment changes have also been studied as potential risk factors for triggering the syndrome. Most cases of NMS arise within 30 days of initiating antipsychotic drug therapy, although some patients may present with symptoms after being treated for several months or even years with the same antipsychotic medication (Mann *et al.*, 2003; Viejo *et al.*, 2003; Tse *et al.*, 2015). Moreover, changes in drug therapy as well as sudden increase of therapeutic dose, discontinuation or resumption of medications, especially if abrupt, are other possible risk factors for the occurrence of NMS. Cases of NMS occurring after antipsychotic withdrawal and after antipsychotics have been changed or reintroduced have been reported (Mann *et al.*, 2003; Pileggi and Cook, 2016).

Lastly, polypharmacy, defined as either the concomitant administration of several antipsychotic drugs or the administration of one or more antipsychotics together with other categories of medications (e.g., lithium carbonate, antiparkinsonian medication, tricyclic antidepressants and benzodiazepines) has been reported as a potential risk factor for the development of NMS (Caroff and Mann, 1993; Mann *et al.*, 2003; Tse *et al.*, 2015).

In conclusion, no risk factors and no adverse effects outweigh the benefits of antipsychotic therapy in psychiatric patients. Although there are many studies on the subject, it is difficult to demonstrate the significance of specific variables in causing NMS, because the number of patients treated in each study is small, sometimes control data are not reported and variables may be correlated and act simultaneously as well as being independent of each other. In addition, because it is a very rare adverse syndrome, it becomes challenging to identify unimpeachable risk factors to predict the occurrence of something as rare as NMS in all populations (Mann *et al.*, 2003).

1.3.6 Genetic risk factors

Although the rare frequency of NMS, case reports of familial occurrence of the syndrome have been reported. It has been reported and suggested, by Otani and colleagues, that the predisposition for susceptibility to NMS may be transmitted genetically, as described for a 43- years-old-mother and her two twenties daughters which developed NMS after antipsychotic treatment, suggesting as a hypothesis that this family was vulnerable to the development of NMS because they shared dysregulation in central dopaminergic systems (Otani *et al.*, 1991; Kawanishi, 2003; Mann *et al.*, 2003; J. R. Strawn, Keck and Caroff, 2007). A few years earlier, Deuschl *et al.* referred the case of two twin brothers with schizophrenia, who developed NMS following drug therapy for the treatment of the psychotic disorder (Deuschl *et al.*, 1987; Mann *et al.*, 2003). Other cases of familial onset have been reported by Manor and colleagues who described the case of two brothers, with gangliosidosis type 2 (a hereditary metabolic disorder) both affected by NMS (Manor *et al.*, 1997).

Finally, a case report of two siblings, a male and a female of 34 and 37 years old, respectively, both schizophrenic and in therapy with risperidone who developed NMS within a week of each other, was also described. In this case, both siblings underwent genetic analysis for three polymorphisms of the D_2 receptor gene (*DRD2*) cited in the literature in correlation with the syndrome (-141C Ins/Del rs1799732, TaqIA rs1800497, Cys311Ser rs1801028). The genetic test revealed that both siblings were carriers of the *del* allele for the polymorphism -141C Ins/Del, an intronic variant classified as INDEL as consists of an insertion or deletion of G (guanine) (Ziegenbein *et al.*, 2006). Indeed, the reference allele G at position chr11:113475530 (forward strand), is changed to GG allele by an insertion of G nucleotide (Ensembl Release 105, GRCh38.p13). This variant has traditionally been named -141C Ins/Del since the gene on which

it is localized was originally mapped in reverse strand and it is characterized by a C insertion on the promoter sequence of the *DRD2* gene.

Moreover, two different groups reported their findings regarding NMS diagnosis and chromosome abnormality: Lazarus *et al.* described a case of NMS in a patient with an inverted duplication of chromosome 5, whereas Rubio-Gozalbo and colleagues, 10 years later, referred a NMS subject, affected by a progressive metabolic disease regarding the nervous system and due to X-linked cerebral adrenoleuko-dystrophy (Lazarus, Moore and Spinner, 1991; Rubio-Gozalbo *et al.*, 2001).

Furthermore, it appears that patients who have developed neuroleptic malignant syndrome once have up to 30% increased risk of recurrence of NMS, especially if they are treated with the same antipsychotic medications (Lazarus, Moore and Spinner, 1991; Perry and Wilborn, 2012; Pileggi and Cook, 2016).

These findings support the theory that genetic predisposition likely represents an important component in the development of NMS, although other elements and risk factors are likely required for the occurrence of this adverse reaction (Rosebush, Stewart and Gelenberg, 1989; Lally *et al.*, 2019).

Pharmacogenetic studies and findings suggest that genetic variations and polymorphisms may be related with interindividual distinctions and/or similarities concerning medication responses, drug efficacy and adverse drug reactions (Evans and Mcleod, 2003).

Currently, several genetic studies have been conducted on the subject, but only a limited number of genetic variants have been selected for association studies with susceptibility to NMS, although with inconclusive and sometimes sharply contrasting results (Kawanishi, 2003; Del Tacca *et al.*, 2005; Živković *et al.*, 2010). Moreover, most of the genetic studies conducted so far were published between the late 1990s and the first decade of the 2000s, mainly restricted to Japanese population while the few data collected for other ethnic groups come mainly from case reports. Therefore, further and more extended investigations are needed to identify genetic biomarkers of susceptibility to NMS (Kawanishi *et al.*, 1998; Kawanishi, 2003; Kishida *et al.*, 2004; Živković *et al.*, 2010).

1.4 DOPAMINE AND DOPAMINERGIC PATHWAY

Dopamine (DA, 3,4-dihydroxyphenethylamine) is a polar molecule and one of the most important neurotransmitters, physiologically located at the level of basal ganglia in the central nervous system, and belonging to the family of catecholamines and phenethylamines. Dopamine is a precursor to norepinephrine and epinephrine in noradrenergic nerves and is an important transmitter in the extrapyramidal system of the brain as well as being most involved in the regulation of movement and a critical modulator of learning and motivation (Rang *et al.*, 2016; Berke, 2018).

In humans, most dopamine biosynthesis is performed directly from tyrosine, but can also be synthesized from phenylalanine absorbed through the diet, which is then converted to tyrosine by the enzyme phenylalanine hydroxylase. The main metabolic pathway occurs in the cytosol and consists of two steps: tyrosine is converted to L-dopa by tyrosine hydroxylase using several cofactors including iron and oxygen; finally, L-dopa is converted to DA by removal of a carboxyl group by L-aromatic amino acid decarboxylase (AADC) using pyridoxal phosphate as a cofactor. L-dopa crosses the blood brain barrier (BBB) easily as opposed to DA, which being a polar molecule does not cross it easily and is subsequently converted to DA in the brain. After the synthesis in dopaminergic neurons, DA is stored until its release into the acidic lumen of synaptic secretory vesicles by the vesicular monoamine transporter 2 (VMAT2) which stabilizes it and prevents its oxidation (Rang *et al.*, 2016; Sibley, Hazelwood and Amara, 2017; Klein *et al.*, 2019).

The metabolism of dopamine is performed both at presynaptic and postsynaptic level by the enzyme monoamine oxidase (MAO) which, through the oxidative deamination of dopamine, generates an inactive aldehydic derivative, afterwards metabolized into 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase. DOPAC can then be metabolized into homovanillic acid (HVA) the major metabolite of DA in humans, by the enzyme catechol-O-methyl transferase (COMT). DOPAC and HVA are accredited markers of DA turnover, accurately representing dopaminergic activity at the brain level. The effects of DA are ended by reassimilation into the presynaptic terminal via the DA transporter (DAT). DAT, located to axons, dendrites and soma of mesencephalic DA neurons, is the target of several stimulant medications and psychostimulants that act by increasing extracellular DA levels within the intrasynaptic compartment. Indeed, euphoric substances such as cocaine act via a blocking effect on the plasma membrane DAT, resulting in increased synaptic DA. Conversely, substances such as amphetamine and methamphetamine, are absorbed into the presynaptic neuron through the DAT, resulting in

increased synaptic amount of DA in the neuronal cytoplasm due to competitive inhibition of re-uptake, which is then released through a non-vesicular mechanism involving outflow through the DAT (**Figure 1.1)** (Rang *et al.*, 2016; Klein *et al.*, 2019).

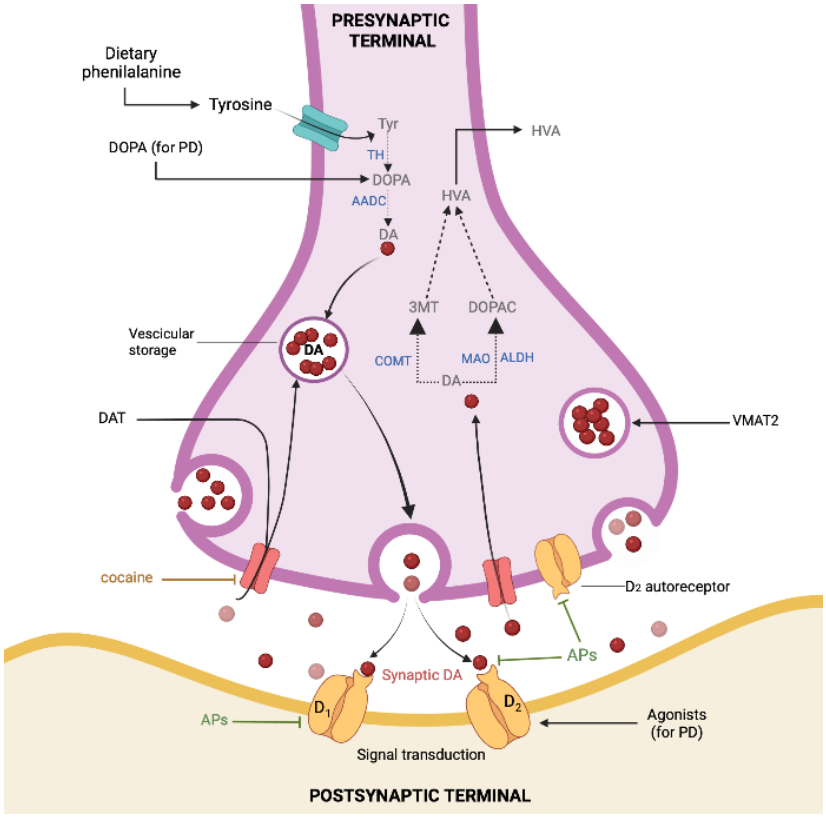

Figure 1.1: Schematic representation of dopaminergic synapse. In figure is detailed the neurotransmitter dopamine and the dopaminergic system. Dopamine is synthesized from tyrosine by the actions of TH and AADC, respectively. DA is sequestered by VMAT2 in storage granules and released by exocytosis. Synaptic DA activates presynaptic autoreceptors and postsynaptic D_1 and D_2 receptors. Adapted from (Sibley, Hazelwood and Amara, 2017).

Abbreviations: 3-MT, 3-methoxytyramine; AADC, L-aromatic amino acid decarboxylase; ALDH, aldehyde

The neurotransmitter dopamine and the dopaminergic system are involved in positive motivational control, reward and non-reward functions, behaviour and mood management, cognitive function, motor control and coordination, body temperature regulation, and neuroendocrine activity. It has been proposed that dopamine neurons, which participate in this multitude of diverse functions, are of several types and are also connected to distinct brain networks with different roles and purposes (Bromberg-Martin, Matsumoto and Hikosaka, 2010; Klein *et al.*, 2019).

The main central dopaminergic pathways in which DA is known to be involved are the nigrostriatal (which originates in the *substantia nigra pars compacta*), the mesocorticomesolimbic

(which originates in the ventral tegmental area) and the tuberoinfundibular pathways (which originates in the hypothalamus). The nigrostriatal pathway is functionally involved in motor regulation, consists of neurons located in the *substantia nigra* that projects into the dorsal striatum and contains more than half of the dopamine present in the brain. Because it is involved in motor control, alterations in dopaminergic transmission in this route lead to the development of Parkinson's disease (PD). In addition, antipsychotic drugs that act by antagonizing dopamine receptors at the level of the nigrostriatal pathway may cause motor adverse reactions in addition to their therapeutic mechanism of action (Rang *et al.*, 2016).

The mesocorticomesolimbic pathway is involved in the emotional control, cognitive function, reward and non-reward function and behavioural management. At the cellular level, neurons belonging to this pathway are localized in the tegmental and ventral area of the midbrain and connects their axons mainly to the *nucleus accumbens* (which belongs to the ventral striatum), to the amygdala and to the frontal cortex. Dysfunction of this pathway is linked to the development of psychosis such as schizophrenia and drug addiction, in part because the effects of some illicit and excitatory substances, such as cocaine and amphetamine, lead to activation of this dopaminergic pathway.

Finally, the tuberoinfundibular pathway includes neurons originating in the hypothalamus that project to the pituitary gland. Dopamine release in this pathway regulates the pituitary gland to secretion and release of prolactin, thereby controlling endocrine function (Rang *et al.*, 2016; Habibi, 2017; Sibley, Hazelwood and Amara, 2017).

1.4.1 Dopamine receptors

Dopamine is not to be considered merely as an excitatory or inhibitory neurotransmitter. In fact, at the physiological level, dopamine can bind to several G-protein-coupled receptors (GPCRs) and modulate adenylate cyclase in different ways depending on the dopamine receptor involved. These were initially categorized in two receptor families: dopamine D_1–like and D_2–like receptors, which are distinguished by their ligand specificities and their effects on G protein-mediated second-messenger systems, as well as on their physiological function and pharmacological properties (Sibley, Hazelwood and Amara, 2017; Klein *et al.*, 2019).

There are five dopamine receptors in mammals, belonging to the two subfamilies:

- D_1–like: belong to this category D_1 and D_5 receptors, encoded by *DRD1* and *DRD5* genes, respectively; they are coupled with G_s/G_{olf} proteins and activates adenylate cyclase (AC) to increase cyclic adenosine monophosphate levels (cAMP);
- D_2–like: belong to this category D_2, D_3 and D_4 receptors, encoded by *DRD2*, *DRD3* and *DRD4* genes, respectively; these receptors are coupled to G_i/G_o proteins which inhibit AC and diminish cAMP production.

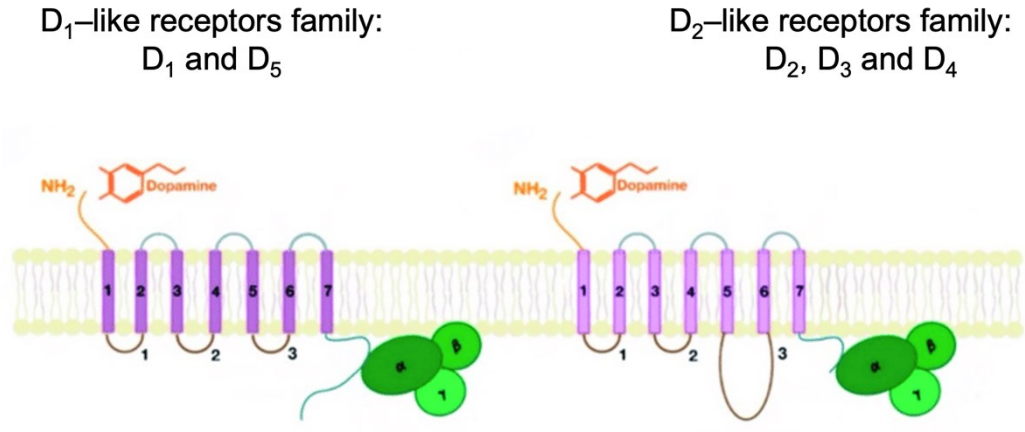

Figure 1.2: Structural differences between D1-like and D2-like family receptors. In the figure is detailed the differenxe between D_1-like and D_2-like family receptors. Adapted from: Scientific Figure on ResearchGate (https://www.researchgate.net/figure/Structural-differences-between-D1-like-receptor-left-side-of-picture-and-D2-like_fig2_3471).

Furthermore, D_2–like receptors can also directly activate potassium channels and inhibit calcium channels through activation of G_i/G_o proteins. Regarding brain localization D_1 and D_2 receptors are present in the limbic system, hypothalamus and striatum at abundant levels, whereas D_2 receptors are also expressed in the pituitary gland, in fact they are involved in the secretion of prolactin that is regulated by dopamine released from the hypothalamus and acting on D_2 receptors present in the pituitary gland (**Figure 1.2**).

The D_1 receptor is the most widely expressed of all 5 dopaminergic receptors. It is located in the kidney, cardiovascular system, retina and of course, at the highest level, in the CNS. The D_1 receptor activates G proteins and, in addition, can form hetero oligomers with ionotropic glutamate NMDA receptors to regulate glutamatergic signalling. The human D_1 receptor gene (*DRD1*) is localized on chromosome 5, possesses only one exon and lacks introns and codifies for a 446 amino acid protein.

The D2 receptor is the second most widely expressed of all 5 dopaminergic receptors. It consists in two main isoforms that originate from alternative messenger RNA splicing: the D_2 short

isoform (D_{2S}) and the D_2 long isoform (D_{2L}). The D_{2S} isoform is a protein 414 aminoacidic residues and lacks 29 amino acids (present in the D_{2L} isoform) in the third intracellular loop. It is present primarily at the presynaptic level, where it acts as a somatodendritic presynaptic autoreceptor to positively or negatively regulate DA synthesis and release in the intrasynaptic area. The D_{2L} isoform, on the other hand, is a slightly longer protein of 443 amino acid residues, and it is present predominantly at postsynaptic level. The two isoforms are pharmacologically alike, both couple to G_i or G_o to diminish cAMP generation. The D_2 receptor is the primary target of many antipsychotic drugs most commonly used in clinical practice, with high binding affinity and specificity. Furthermore, the development of some neurological and neurodegenerative diseases is related to the altered density of this important receptor.

The D_3 receptor is expressed to a lesser extent than D_1 and D_2 receptors. It is encoded by the dopamine receptor D_3 gene (*DRD3*) on the chromosome 3 and produces a protein of 400 amino acids. It is mainly present in the limbic system, more detailed in the *nucleus accumbens*, in the *substantia nigra pars compacta* and in the ventral tegmental area and it is also found in the islands of Calleja. The D_3 receptor, as well as D_{2S}, is also thought to act as an autoreceptor, thereby regulating dopamine release from the presynaptic terminal; it also acts by signalling through G_i/G_o proteins, though not as efficaciously as the D_2 receptor. The D_3 receptor, similarly to the D_1 and D_2, is also thought to be involved in the regulation of emotion and cognition, reward mechanisms and motor control, contributing to the modulation of the D_2 receptor properties, albeit to a lesser extent.

The D_4 receptor is expressed to a lesser extent and is found primarily in the limbic system, in the prefrontal cortex, in the retina, amygdala, hippocampus and hypothalamus. The D_4 receptor consists of a protein of 419 amino acid residues encoded by a dopamine receptor D_4 gene (*DRD4*) located on chromosome 11, that is highly polymorphic, and which includes a variable number of tandem repeats (VNTR) within the third intracellular loop. In humans, the seven-repeat VNTR variant of the D_4 receptor has been associated with the development of attention deficit/hyperactivity disorder (ADHD).

The D_5 receptor is the most widely expressed of all 5 dopaminergic receptors in the hippocampus, but it is also present in hypothalamus, striatum, in the *substantia nigra*, *nucleus accumbens*, cerebral cortex and olfactory tubercle. The D_5 receptor is encoded by the dopamine receptor gene (*DRD5*) located on chromosome 4 and, like the D_1 receptor belonging to the same family, is devoid of introns. The activity of the D_5 receptor, with regard to the functional aspect, is similar to that of the D_4 receptor and is restricted to the modulation of some hippocampus-

mediated cognitive functions (**Table 1.1**) (Bromberg-Martin, Matsumoto and Hikosaka, 2010; Rang *et al.*, 2016; Sibley, Hazelwood and Amara, 2017; Klein *et al.*, 2019).

DA receptor	Subfamily	Gene	Genomic location (strand)	Isoforms (aa length)	CNS expression regions	Known functions	MIM morbidity
D1	D_1-like	DRD1	5:175440036-175444182(-1)	446	widely expressed	activates G proteins; regulate glutamatergic signalling	* 126449
D2	D_2-like	DRD2	11:113409605-113475691(-1)	D2 short (414) D2 long (443)	widely expressed in presynaptic neurons	positively or negatively regulate DA synthesis	* 126450
D3	D_2-like	DRD3	3:114127580-114199407 (-1)	400	limbic system, nucleus accumbens, substantia nigra pars compacta, ventral tegmental area, islands of Calleja	regulation of emotion and cognition, reward mechanisms and motor control	# 181500; # 190300;
D4	D_2-like	DRD4	11:637269-640706(1)	419 highly polymorphic by VNTR variants	limbic system, prefrontal cortex, retina, amygdala, hippocampus, hypothalamus	cognitive functions	# 143465
D5	D_1-like	DRD5	4:9781634-9784009(1)	477	hypothalamus, striatum, substantia nigra, nucleus accumbens, cerebral cortex, olfactory tubercle	hippocampus-mediated cognitive functions	# 606798; # 143465

Table 1.1: Summary of DA receptors. In the table gene, genomic location, protein isoforms, main CNS areas of expression, known CNS function and associated MIM morbidity are listed.

Although DA exerts its activity predominantly at the level of the CNS, it is also involved in some peripheral activities. At the vascular level, circulating dopamine at low concentrations stimulates D_1 receptors resulting in vasodilatation, reducing blood pressure and elevating cardiac contraction. When circulating concentrations of dopamine increase, therefore, DA can activate β-adrenergic receptors to further increase cardiac contractility, whereas at even higher levels, circulating DA activates α-adrenergic receptors resulting in vasoconstriction and increased blood pressure.

Dopamine, in the kidney, is a paracrine/autocrine transmitter and acts on receptors belonging to both the D_1–like and D_2–like subfamilies. At the renal level, DA acts prevalently by augmenting natriuresis, but can also increase glomerular filtration and renal blood flush. Dopamine can also activate $D_1$and D_3 dopaminergic receptors in the kidney with opposite effects, augmenting or diminishing renin secretion, respectively. Dysregulations involving the dopaminergic system and its receptors have been found to be one of the causes of hypertension in humans.

In the pituitary gland, dopamine regulates prolactin secretion. The decrease in prolactin secretion is due to DA, which, after being released by the hypothalamus into the bloodstream, acts

on the D_2 receptors of lactotrophs. Lastly, both D_1 and D_2 receptors are implicated with modulatory action in the release of catecholamines, such as epinephrine (EPI) and norepinephrine (NE). The D_2 receptor stimulation inhibiting NE and also EPI release from sympathetic nerve terminals and from adrenal medulla chromaffin cells respectively, whereas conversely the D_1 receptor stimulation enhances catecholamine release from the adrenal medulla (Sibley, Hazelwood and Amara, 2017).

1.5 ETIOPATHOGENESIS

Despite all the studies conducted on the argument, at present the precise pathophysiology underlying NMS remains relatively unknown, although most authors agree that blockade of dopaminergic activity in central nervous system may be primarily responsible for the onset of neuroleptic syndrome.

It is commonly believed that the antagonism on dopaminergic D_2 receptors in the central nervous system activates a series of homeostatic responses that lead to increased temperature and hyperthermia, cause muscle stiffness and compromise mental status as a result of autonomic nervous system dysregulation. Recently, it has been hypothesized as an alternative that NMS may be due to a toxic effect of pharmacological substances on musculoskeletal fibers, leading only later to the proper syndrome. These two main hypotheses, however, may not be mutually exclusive (Gurrera, 1999; Tse *et al.*, 2015).

According to a study published by Gurrera, the pathophysiological basis of neuroleptic malignant syndrome is due to dysregulation of the sympathetic nervous system hyperactivity. This hypothesis is due to a premise, which is the intrinsic capacity of the sympathetic nervous system to function autonomously and to be involved in all physiological processes relevant to the neuroleptic malignant syndrome (Gurrera, 1999) (**Table 1.2**).

From a study published in 2010, it is suggested that complicated alterations in the functioning of several neurotransmitters within the central nervous system (CNS), such as serotonin (5HT), norepinephrine (NE), glutamate, GABA and in particular dopamine (DA) hypofunction, may play a role in the onset of the syndrome. However, another different approach regarding the pathophysiology of NMS emphasizes hyperactivity of the peripheral sympathetic system associated with the active phase of NMS (Ananth *et al.*, 2004; Margetić and Aukst-Margetić, 2010).

Accordingly, all antipsychotics medication triggering NMS act as antagonists at dopamine receptors in the brain, and sudden withdrawal of dopamine agonists occasionally causes events indistinguishable from NMS in patients with Parkinson's disease who have no antipsychotic exposure. Other neurotransmission pathway, such as the serotonergic and cholinergic systems, have been considered contributors to NMS, either independently or together with the dopaminergic system (Caroff and Mann, 1993; Velamoor, 2017).

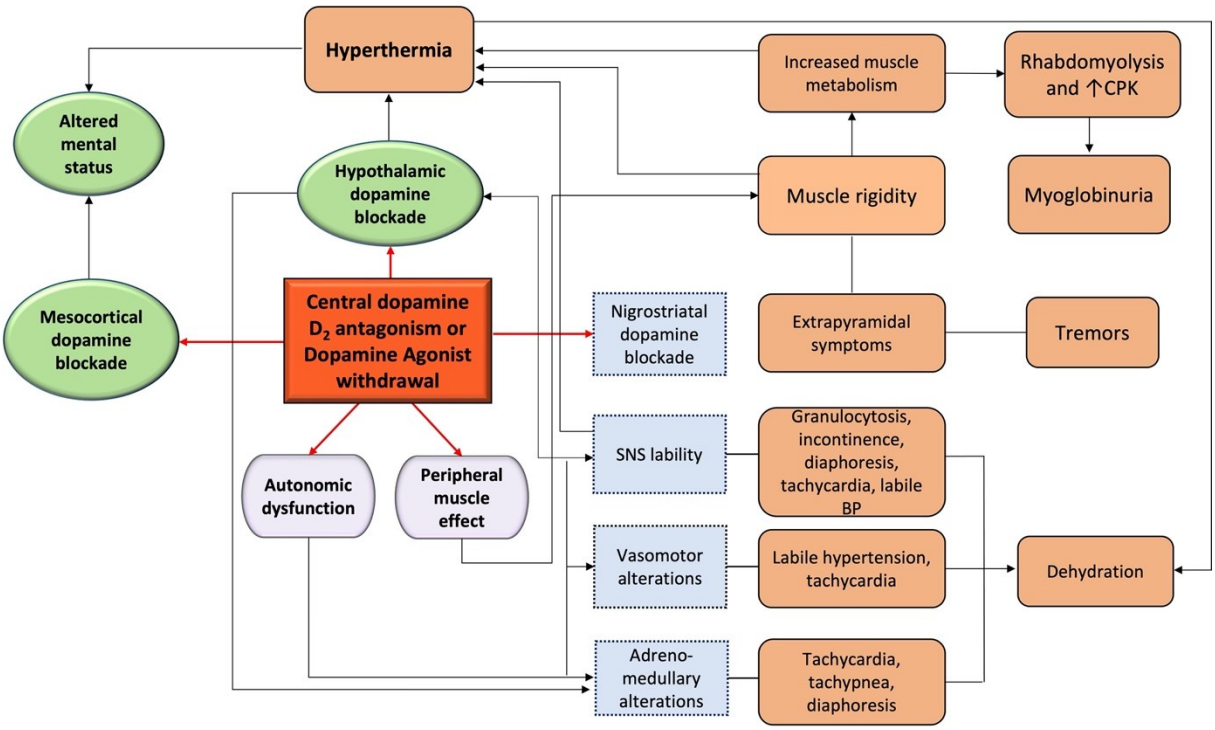

Table 1.2: A semplified entity-relationship model of pathophysiologic mechanisms underlying NMS. Clinical manifestation and elements of autonomic dysfunction are also detailed. Abbreviations: BP, blood pressure; CPK, creatinine phosphokinase; SNS, sympathetic nervous system. Adapted from Gurrera, 1999; Strawn, Keck and Caroff, 2007; Tse et al., 2015.

Mechanisms underlying NMS are not yet clear, however reduced levels of dopamine metabolite in the cerebrospinal fluid (CSF) of patients with acute NMS or lack of D_2 receptor binding activity in a patient with acute NMS have been described, supporting the hypothesis that a blockade of dopamine receptor D_2 (*DRD2*) in the brain may be a critical step for the onset of the syndrome (Jauss *et al.*, 1996; Mann *et al.*, 2000).

1.5.1 Dopaminergic receptor hypofunction hypothesis

Dopamine neurotransmission is vitally involved in regulation of body temperature, which is mediated in the thermoregulatory center of the hypothalamus, especially in the anterior preoptic nucleus. Consequently, first-generation antipsychotics, which act as antagonists of dopamine receptor-mediated signaling on neurons in the thermoregulatory center region, may lead to dysregulation of the thermoregulatory mechanisms (Henderson and Wooten, 1981; Tse *et al.*, 2015).

Several cases described in literature, concerning patients with Parkinson's disease who developed NMS following the abrupt discontinuation of treatment with dopamine agonist drugs and subsequently developed NMS, are consistent with the theory that disruption of dopamine receptor-mediated signaling is likely a mechanism leading to the syndrome. In addition, cases concerning NMS patients in treatment with catecholamine-depleting drugs provide additional demonstration that dysregulation of dopamine signaling is the primary mechanism underlying the development of NMS. In fact, hyperthermia, one of the main features of NMS due to a lack of dopaminergic signaling in the thermoregulatory system, can be caused by a sudden decrease in postsynaptic receptor stimulation or postsynaptic receptor blockade, as well as by a lack of neurotransmitter itself (Henderson and Wooten, 1981; Mann *et al.*, 2003; Ananth *et al.*, 2004; Tse *et al.*, 2015; Pileggi and Cook, 2016).

Several studies have been conducted to shed light on the etiopathogenesis of NMS. In a report published in 1995, Nisijima and Ishiguro measured the level of various cerebrospinal fluid (CSF) in 11 NMS patients and compared them with 8 age-matched normal controls without neuro-psychological problems. The mean ages of the NMS and the control group were 35.2 + 11.6 years and 39.1 + 9.6 years, respectively. All cases met the diagnostic criteria for NMS developed by Levenson (1985). As for the GABA level, 8 NMS patients were compared with an equal number of control subjects. The mean ages of the NMS group and control group were 37.9 + 11.4 years and 34.1 + 10.4 years, respectively.

Depending on the subjects, the study was performed from one to several times, from the active phase of NMS to the period after improvement. The measurement of the values in the present study was limited to samples taken before the administration of therapeutic drugs during the active phase of NMS in all cases, because drugs such as dantrolene and bromocriptine can influence the results, while the measurement of values after improvement was limited to the period before re-administration of antipsychotics in all cases.

CSF investigated parameters were homovanillic acid (HVA), serotonin's main metabolite 5-hydroxyindoleacetic acid (5-HIAA), noradrenaline (NA) and its metabolite 3-methoxy-4-hydroxy-phenylethyleneglycol (MHPG), and gamma-aminobutyric acid (GABA).

HVA levels were significantly decreased during the active phase of NMS and after the recovery phase, compared with levels in normal subjects. Concentrations of 5-HIAA were also decreased in the active phase, however not significant. 5-HIAA levels after recovery were significantly decreased compared with controls. Concentrations of noradrenaline and its metabolite (MHPG)

were significantly higher during the active phase of NMS compared with controls, but returned to parameters after recovery. The levels of GABA during the active phase and after recovery were significantly lower than in the controls, although they had a tendency to increase from the active phase to the recovery phase of NMS. A significant and positive correlation was noted between HVA and 5-HIAA levels, and NA and MHPG levels. In contrast, there was no correlation between the levels of HVA or 5-HIAA and body temperature or pulse rate, while a significantly positive correlation was noted between the level of NA and body temperature or pulse rate. Moreover, a significantly correlation was also found between the level of MHPG and body temperature or pulse rate. Finally, the CSF level of GABA was significantly low in the active phase and after improvement compared with the level in the control group (Nisijima and Ishiguro, 1990, 1995).

These findings support the central dopaminergic blockade theory of NMS. Although a relationship between the development of NMS and abnormality in the serotonin system remains unclear, it is suggested that there is hyperactivity in the noradrenaline system and hypoactivity in the GABAergic system. Hyperactivity of the noradrenaline system may be clinically related to autonomic nervous symptoms such as hyperthermia, tachycardia, etc. At present, it is not yet certain whether the levels of NA or MHPG are secondary to the clinical symptoms or whether hyperactivity of noradrenaline system is directly related to the development of NMS. Thus, in addition to an abnormality in the dopamine system, various nervous systems are affected in NMS, and this may be related to the variety of clinical symptoms typical of NMS (Nisijima and Ishiguro, 1990, 1995).

These results are nearly consistent with those reported in a previous study by Kish and colleagues, who performed biochemical analysis of the brains of three patients, two of whom died of fatal catatonia and one of whom died of neuroleptic malignant syndrome by observing a marked reduction - from 50% to 70% - of HVA levels in the striatum in two of the patients, one fatal catatonia patient and the NMS patient. This suggests that the impaired ability of the nigrostriatal dopaminergic system to properly respond to stress and/or neuroleptic-induced receptor blockade, also exacerbated by cholinergic deficiency, may be relevant in the occurrence and progression of neuroleptic malignant syndrome as well as in fatal hyperthermia syndrome (Kish *et al.*, 1990).

In another study, De Reuck and his group investigated the determination of blood flow and oxygen metabolism by Positron Emission Tomography (PET) in 3 patients with NMS showed an increase of regional blood flow (rCBF) and oxygen consumption (rCMRO2) in striatum, cerebellum and occipital cortex for two of them, providing evidence for functional alterations of the dopaminergic system during NMS (De Reuck *et al.*, 1991).

Furthermore, imaging of dopamine receptors occupancy performed with Single-Photon Emission-Computed Tomography (SPECT) using the radioactive ligand [123I]Iodobenzamide (IBZM), which has a high binding affinity to D_2 receptors on a patient in the acute phase of NMS and during the course of remission from the syndrome showed an almost complete occupation of D_2 receptors during NMS and a reduced IBZM binding persisting still after 3 months after NMS onset (Jauss *et al.*, 1996).

Moreover, dopamine neurotransmission alteration in the basal ganglia, a subcortical nuclei group that regulate muscle tone and motor coordination, may explain other NMS typical symptoms. The pathophysiology of Parkinson's disease (PD), where neurodegeneration of the dopaminergic system located in the substantia nigra of the midbrain causes a loss of dopaminergic transmission that results in increased muscle tone, increased stiffness and tremor, is further evidence of a role of disrupted dopaminergic signaling in the etiology of NMS. Therefore, Parkinson's disease patients are treated with drug therapy with agonist action on dopamine receptors to reestablish dopaminergic signaling and improve clinical manifestations. In contrast, subjects in therapy with first-generation antipsychotics may develop Parkinson's-like symptoms: rigidity, tremor and improved muscle tone. Hence, it is hypothesized that the clinical manifestations observed in NMS such as rigidity, tremor, and hypertonia are therefore due to the blockade of dopamine receptors in the basal ganglia (Ossowska, 2002; Tse *et al.*, 2015).

Moreover, the possibility that second-generation antipsychotics (like clozapine, risperidone and quetiapine) or antipsychotic medications administered in low doses may cause NMS, suggests that the syndrome may also be induced by substances that have weak dopamine receptors antagonism (Ananth *et al.*, 2004; Margetić and Aukst-Margetić, 2010).

The development of NMS after a therapy with antipsychotic drugs whose proposed mechanisms of action are different from traditional antipsychotics, such as aripiprazole which is a partial dopamine D_2 receptor agonist instead of antagonist, makes the comprehension of the pathophysiological mechanism of the syndrome even more challenging. Therefore, it remains unclear whether the dopaminergic system is the one and only involved in the development of NMS (Margetić and Aukst-Margetić, 2010).

1.5.2 Other neurotransmitter systems hypothesis

Although the pathophysiologic mechanism involving blockade of the dopaminergic system seems to be the most widely accepted hypothesis for the occurrence of NMS, many authors have proposed several alternative factors that may contribute to the development of the syndrome.

Weller and Kornhuber before (1992a) and Kornhuber and colleagues after (1993) proposed that NMS can be considered an iatrogenic syndrome consequent to an excess of relative glutaminergic transmission occurring as a result of dopaminergic blockade given by the action of antipsychotic drugs (Weller and Kornhuber, 1992; Kornhuber, Weller and Riederer, 1993; Mann *et al.*, 2003).

Other authors have hypothesized that low serum iron levels may be risk factors or markers associated with NMS. Specifically, Rosebush and colleagues (1991) and then J.W.Y. Lee a few years later (1998) reported that poor iron levels in NMS patients may participate in the onset of the syndrome because iron may have a key role in the regular function of central dopamine receptors D_2 (Rosebush, Stewart and Mazurek, 1991; Lee, 1998; Mann *et al.*, 2003).

Furthermore, in a review conducted by Gurrera it was assumed that drug-induced discontinuation of central inhibitory inputs leads to peripheral sympathetic system hyperactivity and dysregulation. The sympathetic nervous system is relative independent from the central nervous system and is regulated by the lateral and posterior hypothalamus and the frontal cortex and its dysregulation leads to an over-stimulation of thermoeffector end organs expressed by augmented muscle tone and metabolism, a deficiency in heat dissipation, raised mitochondrial thermogenesis, oscillations in vasomotor tone, urinary incontinence and granulocytosis (Gurrera, 1999; Mann *et al.*, 2003; Margetić and Aukst-Margetić, 2010).

Lastly, the hypothesis that NMS may be a condition due to a toxicity of the musculoskeletal fibers is supported by evidence that NMS and malignant hyperthermia (MH) are two very similar adverse reaction. Malignant hyperthermia is a very rare condition that occurs in predisposed individuals following administration of halogenated anesthetics. It is characterized by a high body temperature, as well as an abnormal muscle contractile response. The abnormal contraction of musculoskeletal fibers in response to the *in vitro* halothane and caffeine exposure test, that characterizes MH subjects, is comparable to biopsies obtained from muscle fibers of NMS patients (Tse *et al.*, 2015).

However, NMS and MH are two very different syndromes. In fact, NMS can occur as an extrapyramidal reaction or catatonic disorders and may also present in milder variation. MH is a less frequent adverse syndrome than NMS, and it also develops abruptly and has a much shorter duration than NMS. The different and sometimes opposite properties that characterize these two syndromes are intrinsically related to the etiopathogenic processes underlying NMS and MH (Keck, Caroff and McElroy, 1995; Mann *et al.*, 2000, 2003). These opposite properties and manifestations are also reflected in the difference that nondepolarizing muscle relaxants have on these syndromes: in fact while in MH patients these substances have no effect, in NMS patients they instead reduce muscle stiffness by impeding neural input to relative receptors (Mann *et al.*, 2003; Rosenberg and Rueffert, 2011; Rosenberg *et al.*, 2015).

The similar clinical manifestations between NMS and MH have raised a legitimate doubt regarding the risk that there may be cross-reactivity between the two adverse reactions. Studies to date, however, would seem to exclude the possibility that NMS patients may be at risk for MH following anesthesia. Malignant hyperthermia is thought to be a rare pharmacogenetic disorder due to calcium dysregulation in skeletal muscle, whereas NMS is considered to be a hypodopaminergic manifestation in the central nervous system (Keck, Caroff and McElroy, 1995; Mann *et al.*, 2003; Rosenberg and Rueffert, 2011; Rosenberg *et al.*, 2015).

1.6 CAUSATIVE AGENTS

Although the precise pathophysiology which subtends NMS has not been precisely identified yet, it is widely well established that its onset is due to exposure to drugs and medications that therapeutically act on the dopaminergic system, and particularly on the dopamine D_2 receptor.

All drugs that have the capability of blocking dopamine receptor, including antipsychotics, some antiemetics (such as metoclopramide) and some tricyclic antidepressants (TCA) have been reported to cause the syndrome. Nevertheless, other categories of medication with different mechanisms of action other than drugs that act by blocking the dopaminergic system have also been reported to cause NMS: among these, lithium, used as a mood stabilizer in therapies for the treatment of depression and to treat and prevent manic episodes in those with bipolar disorder, clomipramine, a tricyclic antidepressant employed in the treatment of obsessive-compulsive disorders and selective serotonin reuptake inhibitors (*SSRIs*), such as citalopram, used to treat the symptoms of depression (J. R. Strawn, Keck and Caroff, 2007; Margetić and Aukst-Margetić, 2010; Simon, Hashmi and Callahan, 2021). Moreover, there are several case reports in literature of NMS caused by the abrupt and sudden suspension of treatment with drugs with dopaminergic and dopamine-agonist action, such as those used for the treatment of Parkinson's disease (levodopa, amantadine, baclofen) (Ananth *et al.*, 2004; Margetić and Aukst-Margetić, 2010; Oruch *et al.*, 2017). Although, according to the last edition of DSM-V (Diagnostic and statistical manual of mental disorders- Fifth edition) NMS patients “have generally been exposed to a dopamine antagonist within 72 hours prior to symptom development” (*Diagnostic and Statistical Manual of Mental Disorders*. 5th edn, 2013) (**Table 1.3**).

Antipsychotic drugs (APs) also known as neuroleptics, are a various class of medication effectively used in the pharmacological treatment of schizophrenia and other psychotic disorders, as well as psychotic syndromes and symptoms in general (Zhang and Malhotra, 2011; Lally and MacCabe, 2015). The main mechanism of action and pharmacodynamic property of all antipsychotics is well known and consists in blocking the dopamine D_2 receptors in the central nervous system acting as antagonists, with higher or lower affinity depending on the category of medication (Lally and MacCabe, 2015). These classes of drugs have been categorized into two subtypes according to their receptor affinity. The first one to be developed, called “typical” or “conventional” neuroleptics, or more commonly first-generation antipsychotics (FGAs) have high affinity for dopaminergic system by blocking D_2 receptors in the mesolimbic system, causing as a

result a decrease in dopamine levels in this district and other dopamine-relative pathways (Naumovska *et al.*, 2015).

First-generation antipsychotics are considered extremely effective for the management of the "positive" symptoms of psychiatric disorders (including hallucinations, psychomotor agitation, hostile and aggressive behavior, speech impairment). However, potent FGAs (or typical neuroleptics) such as haloperidol, chlorpromazine, fluphenazine and trifluoperazine among others, have been most recurrently associated with the development of NMS and are also thought to carry the highest risk (Brian D Berman, 2011). Moreover, this group of medication is linked to the development of several adverse effects, mainly dopamine blockade-induced movement disorders such as extrapyramidal symptoms (EPS), parkinsonism, dystonia, akathisia and dyskinesia (acute or tardive) as well as other metabolic side effects such as weight gain and prolactin changes (Mas *et al.*, 2016; Patton and Borshoff, 2018). EPSs are primarily dose-related among patients using first-generation antipsychotic drugs and are the most frequent adverse effects with an incidence of 76% in adults (Sacristán *et al.*, 2000; Pike *et al.*, 2009).

These severe and debilitating side effects associated with FGAs lead to the need for additional pharmacotherapy and, often, are the cause of poor adherence to psychiatric therapy by individuals who are already vulnerable because of their illness, with negative consequences for the patients themselves and their families. For these reasons, in the early 1990s were developed other categories of antipsychotics drugs with the aim of minimizing or abolishing these stigmatizing adverse effects (Lally and MacCabe, 2015; Mas *et al.*, 2016).

Newer second-generation antipsychotics (SGAs) formerly known as “atypical” neuroleptics, have a different mechanism of action: in addition to acting as dopamine D_2 receptors antagonists, which they share with their predecessors FGAs, they also have a mainly affinity for 5-HT serotonergic receptors, and also for histaminergic, cholinergic, and adrenergic receptors. These characteristics contribute to the fact that patients in treatment with SGAs are less likely to develop motor adverse reactions but are prone to developing metabolic side effects such as obesity, diabetes, dyslipidemia, weight gain, hypertension, cardiovascular diseases and other dysfunctions (Pike *et al.*, 2009; Zhang and Malhotra, 2011; Naumovska *et al.*, 2015). Although SGAs, originally thought to be even exempt from the risk of causing NMS, would appear to have reduced the risk of developing NMS following their use compared with first-generation antipsychotics, a significant number of NMS cases have been reported with many of the usually prescribed atypical antipsychotics, including, among others, aripiprazole, clozapine, quetiapine and risperidone (Brian D Berman, 2011; Belvederi Murri *et al.*, 2015; Oruch *et al.*, 2017).

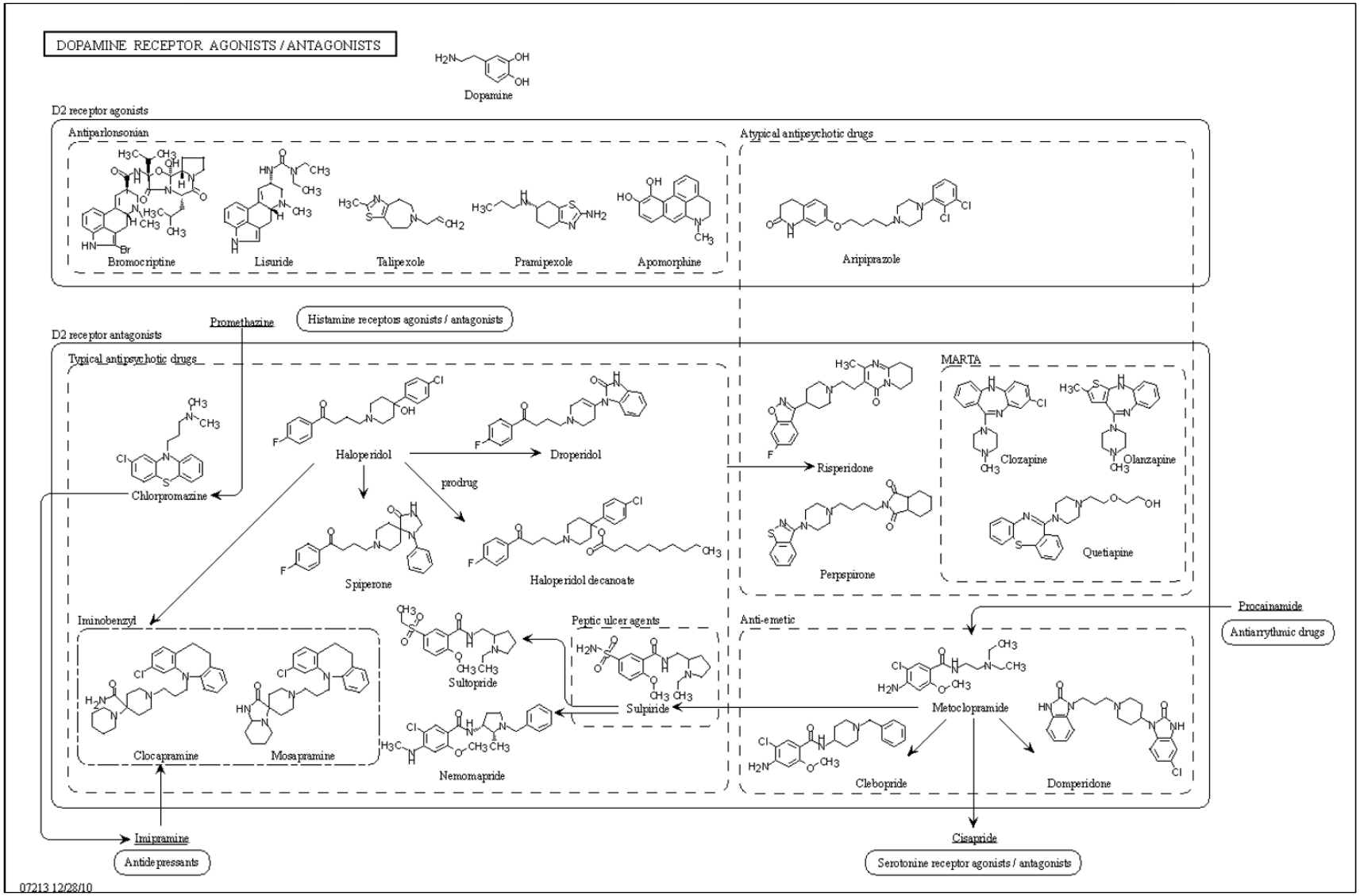

Table 1.3: Dopamine receptor agonists and antagonists. In the figure are reported dopamine D_2 receptor agonists and antagonists names and structures, including certain NMS causative agents. Arrows indicate links among drug structures. Adapted from: https://www.genome.jp/dbget-bin/www_bget?map07213).

Other categories of drugs, such as some antiemetics and gastroprokinetics (e.g., promethazine, domperidone, metoclopramide), some antidepressants (e.g., amoxapine, citalopram), some drugs that act as anesthetics and antiemetics (e.g., droperidol) are often used medically as neuroleptic medications, and have also been associated with the development of NMS in treated patients (**Table 1.4**) (Ware, Feller and Hall, 2018).

FIRST-GENERATION ANTIPSYCHOTICS	SECOND-GENERATION ANTIPSYCHOTICS	ANTIEMETICS	ANTIDEPRESSANTS	DOPAMINERGICS (WITHDRAWAL)	OTHERS
Chlorpromazine	Aripiprazole	Domperidone	Amoxapine	Amantadine	Lithium
Fluphenazine	Clozapine	Droperidol	Citalopram	Baclofen	Phenelzine
Haloperidol	Olanzapine	Metoclopramide	Desipramine	Dopamine agonists	Dosulepin
Loxapine	Paliperidone	Prochlorperazine	Dosulepine	Levodopa	Desipramine
Mesoridazine	Quetiapine	Promethazine	Phenelzine	Tolcapone	Trimipramine
Molindone	Risperidone				Valproate
Perphenazine	Ziprasidone				
Promazine					
Thioridazine					
Zuclopenthixol					

Table 1.4: Causative agents. In the table is reported a list of some drugs and medications, divided in different categories, associated with Neuroleptic Malignant Syndrome. Adapted from Berman, 2011; Wilson et al., 2016; Oruch et al., 2017.

1.6.1 Haloperidol

Haloperidol is a high potency first-generation antipsychotic (FGA) indicated for the treatment of schizophrenia and several other psychotic disorders (**Figure 1.3**). It is also prescribed to manage delirium, agitation, irritability, as a potent antiemetic, and it is also prescribed off-label for the treatment of chorea associated with Huntington's disease (Dold *et al.*, 2015). It is one of the most commonly prescribed and used antipsychotic drugs worldwide (Adams *et al.*, 2013).

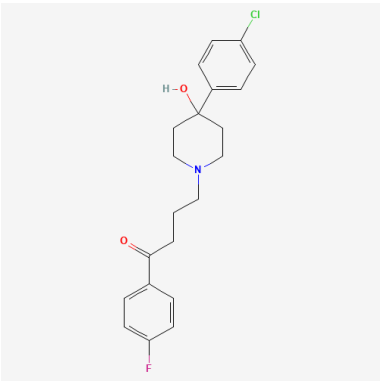

Figure 1.3: Haloperidol structure. A two-dimensional representation of the compound is detailed. From https://pubchem.ncbi.nlm.nih.gov/compound/3559.

Haloperidol performs its pharmacological activity on different receptors in the brain, but mainly exerts its antipsychotic effect through its strong antagonism with dopamine receptors (primarily D_2), particularly in the mesolimbic and mesocortical districts of the central nervous system (Kroeze *et al.*, 2003). It has been estimated that the optimum clinical efficacy of antipsychotics medication is related to the blockage of about 60% to 80% of D2 receptors in the brain (Seeman and Kapur, 2000).

1.6.1.1 Mechanisms of action

Drugs that act as dopaminergic receptor antagonists -such as haloperidol- have been created to ameliorate psychotic symptoms and situations that are caused by a dysregulation in dopamine release, such as schizophrenia, which results in an overproduction and overrelease of dopamine that is hypothesized to be induced by a hyperdopaminergic state in the limbic system of the brain (Seeman and Kapur, 2000).

It is now established that haloperidol acts by inhibiting the effects of dopamine and increasing its turnover, although the precise mechanism of action of this antipsychotic remains partially misunderstood. What is known about traditional or first-generation antipsychotics, however, is that they bind more tightly to the dopamine receptor than dopamine itself, with dissociation constants lower than those of dopamine (Seeman, 2002).

Haloperidol works by suppressing dopamine neurotransmission in order to reduce or manage the hallucinations and delusions that are typically associated with psychosis, primarily through competitively blocking post-synaptic dopaminergic receptors D_2 in the brain. It also acts on 5-HT2 and α1 receptors, on dopamine D_1-receptors with marginal consequences, and also exerts a blocking action against α-adrenergic receptors in the autonomic nervous system (Dold *et al.*, 2015). Antipsychotic drugs block only the D_2 receptor of the three belonging to the D_2-like receptors group, which includes also D_3 and D_4.

It is widely known, thanks to positron emission tomography (PET) studies carried out on human patients, that haloperidol remains tightly bound to dopamine D2 receptors. This close link causes in many cases a common but disabling adverse effect: extrapyramidal symptoms (EPS) (Seeman, 2002).

The risk of developing these debilitating and lifelong adverse effects led pharmacologists to develop and formulate new antipsychotic drugs, like the atypical antipsychotic Risperidone, which

had the ability to rapidly dissociate from dopamine D2 receptors as their main feature (Seeman, 2002).

1.6.1.2 Pharmacodynamics

Although first-generation antipsychotics, which include haloperidol, are considered extremely efficient at managing the positive symptoms of schizophrenia, such as visual and auditory hallucinations, agitation, aggression behaviors, disorganized language and hostility, their use has been progressively limited over time because of their association with the development of metabolic, motor, and other less severe adverse effects (Adams *et al.*, 2013; Tardy *et al.*, 2014; Dold *et al.*, 2018).

Haloperidol typically determines a lower propensity to cause side effects among first-generation antipsychotic drugs, especially when compared with low-potency FGAs such as zuclopenthixol chlorpromazine methotrimeprazine and fluphenazine, but is more frequently the causative agent for the development of extrapyramidal symptoms. The treatment of schizophrenia's symptoms with low potency antipsychotics requires higher doses of therapy because these medications have a reduced affinity for the primary dopamine receptors target and, in addition, they block many receptors than D_2, for example histaminergic and cholinergic and consequential increase in adverse effects, such as hypotension, sedation and weight gain (Adams *et al.*, 2013; Tardy *et al.*, 2014; Dold *et al.*, 2018).

It is the very action of haloperidol on dopaminergic pathways in the brain that produces both the therapeutic effects on the symptoms of psychosis and the undesirable and debilitating adverse effects. Cortical dopamine-D_2 pathways have significant responsibility in triggering these effects: extrapyramidal symptoms (EPS) are related to the nigrostriatal pathway, the improvement of positive symptoms of schizophrenia are due to the mesocortical and mesolimbic pathways, whereas hyperprolactinemia is related to the tuberoinfundibular dopamine pathway (Beresford and Ward, 1987).

1.6.1.3 Metabolism

In human, haloperidol is widely metabolized at hepatic level and biotrasformed in different metabolites: p-fluorobenzoylpropionic acid, 4-(4-chlorophenyl)-4-hydroxypiperidine, haloperidol glucuronide (which is the metabolite with the highest concentration in plasma of patients regularly treated with haloperidol) pyridinium metabolites and reduced haloperidol (**Figure 1.4**).

The biotransformation of haloperidol occurs from enzymes that are members of cytochrome P450 (*CYP*) amongst *CYP3A4* and *CYP2D6*, as well as carbonyl reductase and uridine di-phosphoglucose glucuronosyltransferase enzymes. Most intrinsic hepatic clearance of haloperidol occurs by glucuronidation followed by haloperidol reduction to reduced-haloperidol and CYP-mediated oxidation.

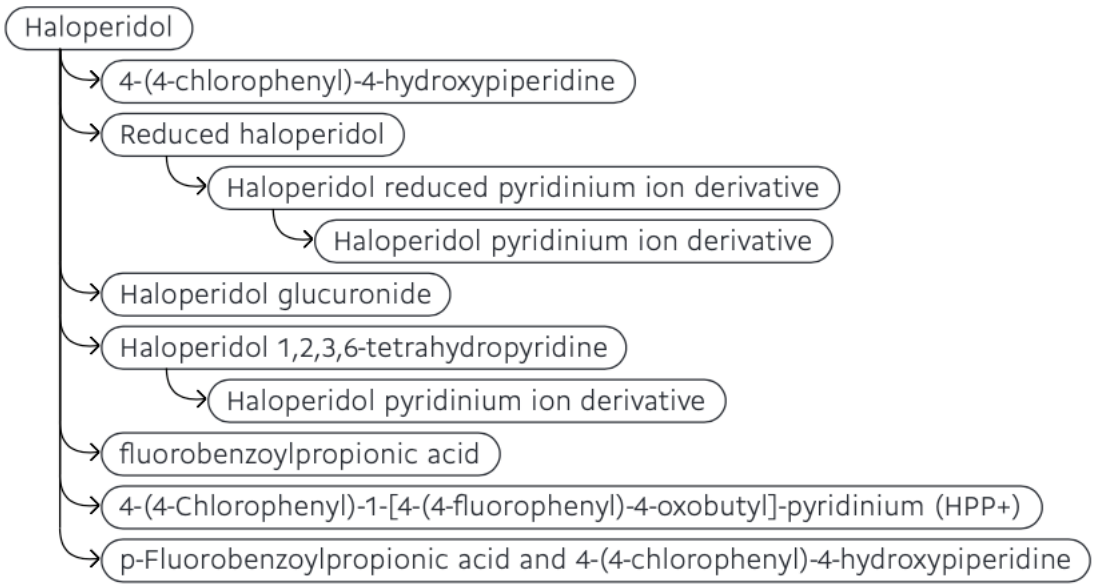

Figure 1.4: Haloperidol metabolism. In the figure the haloperidol metabolism is detailed. Haloperidol is extensively metabolized in the liver with only about 1% of the administered dose excreted unchanged in urine.

CYP3A4 seems to be the main enzyme isoform involved in haloperidol metabolism in humans, thanks to studies conducted in vitro on cytochrome-mediated disposition. Other studies conducted in humans in vivo showed that haloperidol glucuronidation is the main route of haloperidol metabolism, responsible for approximately 50-60% of haloperidol biotransformation in vivo and that approximately 23% of the biotransformation was represented by the reduction pathway. The residual 20-30% of haloperidol biotransformation would occur via N-dealkylation and pyridinium formation (Kudo and Ishizaki, 1999).

1.6.2 Promazine

Promazine is a first-generation antipsychotic that belongs to the group of phenothiazine antipsychotic drugs, which have similar action to chlorpromazine but with less antipsychotic activity (**Figure 1.5**). It is used primarily in the short-term treatment of disturbed behavior and as an antiemetic, but also as an adjunct for the short-term treatment of moderate to severe psychomotor agitation and to treat psychosis, schizophrenia, violent aggressive behavior. In addition, it is prescribed to senior patients to treat agitation or restlessness.

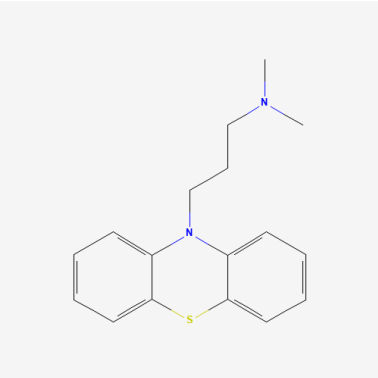

Figure 1.5: Promazine structure. A two-dimensional representation of the compound is detailed. From https://pubchem.ncbi.nlm.nih.gov/compound/4926

1.6.2.1 Pharmacodynamics

Promazine medication acts by blocking several types of receptors in the brain, mostly dopamine receptors. In fact, dopamine is implicated in signals transmission between neurons, but an excess of this neurotransmitter in the brain leads to overstimulation of dopamine receptors, a condition that can lead to the development of psychotic illness. Promazine hydrochloride blocks these receptors and prevents them from being overstimulated, thus helping to manage psychosis. However, its antipsychotic effect is limited and is not very helpful in general psychiatry. In addition, promazine induces weak extrapyramidal and autonomic side effects, leading to its use in older subjects, for restless or psychotic patients.

1.6.2.2 Mechanism of action

Promazine is an antagonist of both dopamine D_1-like (*DRD1*) and D_2-like receptors (*DRD2* and *DRD4*), 5-HT_{2A} and 5-HT_{2C} receptor types, alpha(1)-receptors, muscarinic receptors 1 through 5 and histamine H1-receptors. Its antipsychotic effect is attributable to greater antagonism for

serotonin 5-HT_2 receptors than for dopamine D_2 receptors, which may explain the absence of extrapyramidal adverse effects. On the other hand, the lower incidence of hyperprolactinemia compared with other typical antipsychotics or risperidone would appear to be due to the failure of promazine to block dopamine from the tubero-infundibular tract.

1.6.3 Zuclopenthixol

Zuclopenthixol is a thioxanthene-based neuroleptic with therapeutic actions similar to phenothiazine antipsychotics. It belongs to the "typical" or first-generation antipsychotics group and is also known as Zuclopenthixolum (**Figure 1.6**). It is use in psychiatric emergencies for the treatment of acute or chronic schizophrenia or the management of acute psychosis and manic episodes as an alternative to standard treatments with haloperidol, clotiapine and other antipsychotics, even if this medication is not intended for long-term use.

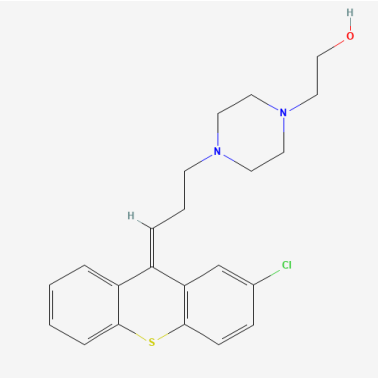

Figure 1.6: Zuclopenthixol structure. A two-dimensional representation of the compound is detailed. From https://pubchem.ncbi.nlm.nih.gov/compound/5311507

1.6.3.1 Pharmacodynamics

Zuclopenthixol is an antipsychotic compound belonging to the thioxanthenes class and it is a short-acting drug. Zuclopenthixol metabolism occurs mainly by sulfoxidation, N-dealkylation of the side chain, and glucuronic acid conjugation. The metabolites are devoid of pharmacological activity.

Although no cases of overdose have been reported, this antipsychotic may mask signs of toxicity from overdoses of other drugs. Related symptoms are likely to be extrapyramidal symptoms, drowsiness, coma, hypotension, seizures, shock, or hyper- or hypothermia. Furthermore, neuroleptic malignant syndrome may occur. Mechanism of action

It acts mostly as an antagonist of dopamine D_1-like receptors (*DRD1, DRD5*) and dopamine D_2 receptors (*DRD2*). Additionally, other targets of this antipsychotic medication involve 5-hydroxytryptamine receptor 2A (*HTR2A*) and alpha-1A adrenergic receptor (*ADRA1A*) with high affinity. Furthermore, it has weaker histamine H1 receptor (*HRH1*) blocking activity and even lower affinity for muscarinic cholinergic and alpha2-adrenergic receptors (*ADRA2A*). Moreover, zuclopenthixol is metabolized by Cytochrome P450 2D6.

1.6.4 Aripiprazole

Aripiprazole is an atypical antipsychotic drug (SGA) used for the treatment of an extensive variety of psychotic and mood disorders, such as schizophrenia, bipolar disorder, Tourette's syndrome (TD), irritability and agitation associated with the autism spectrum; additionally, it is administered as an adjunctive treatment of major depressive disorder (MDD) (**Figure 1.7**).

It is metabolized predominantly in the hepatic district in its primary metabolite dehydroaripiprazole, by *CYP3A4* and *CYP2D6* (Gupta and Masand, 2004; Molden *et al.*, 2006).

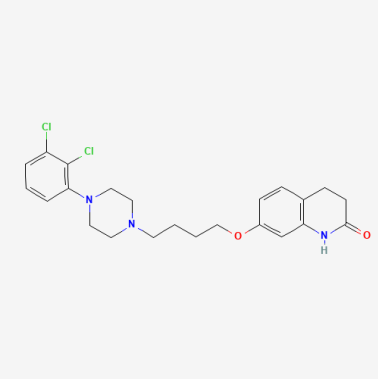

Figure 1.7: Aripiprazole structure. A two-dimensional representation of the compound is detailed. From https://pubchem.ncbi.nlm.nih.gov/compound/60795

1.6.4.1 Pharmacodynamics

At pharmacodynamic level it acts with high affinity as an antagonist at serotonin 5-HT_{2A} receptors (*HTR2A*) and as an antagonist and partial agonist of dopamine D_2 receptors (*DRD2*), in addition to being a partial agonist of serotonin 5-HT_{1A} receptors (*HTR1A*). Furthermore, aripiprazole is an antagonist at alpha1 and 2 adrenergic (*ADRA1A* and *ADRA2A*) and H1 histaminergic receptors (*HRH1*), it has high and moderate affinity for dopamine D_3 (*DRD3*) and

dopamine D_4 (*DRD4*) receptors respectively and also for *HTR2C* and *HTR7*. (Aihara *et al.*, 2004; Argo, Carnahan and Perry, 2004).

Moreover, aripiprazole acts with minor affinity on other receptors, nevertheless the exact method by which the effect of aripiprazole on these receptors translates into a clinically relevant response is not known yet.

1.6.4.2 Mechanism of action

Although the precise mechanism of action of aripiprazole has not been defined, it is most probable that its therapeutic effect is due to D_2 and 5-HT_{1A} receptors partial agonism. It is known that aripiprazole stabilizes dopamine and serotonin activity in the limbic and cortical system.

Some adverse effects, such as extrapyramidal symptoms, especially among neonates and children, or orthostatic hypotension, and may be explained by the antagonism action on adrenergic alpha1 receptors and others (Nasrallah, 2008).

Polymorphisms associated with *DRD2* and *ANKK1* genes were investigated in involvement with aripiprazole efficiency in schizophrenic patients (Kim *et al.*, 2008; Kwon *et al.*, 2008; Shen *et al.*, 2009). Moreover, a pharmacogenetic study conducted *in vitro* demonstrated that several genetic variants of *HTR2A* gene were related with alterations in the potency of aripiprazole and three other atypical antipsychotic drugs (clozapine, quetiapine, and risperidone) at the cellular level (Davies, Conley and Roth, 2011).

1.6.4.3 Metabolism

In human, aripiprazole is predominantly metabolized at hepatic level, mostly by cytochrome P450 *CYP3A4* and *CYP2D6*. These enzymes perform dehydrogenation and hydroxylation while CYP3A4 alone performs N-dealkylationLabel. At any given time, the active metabolite dehydro-aripiprazole is approximately 40% of the drug available in plasma (Bauman *et al.*, 2008).

The half-life of aripiprazole is usually 75 hours, but it has been found that in patients classified as CYP2D6 poor metabolizers it has a half-life of 146 hours, so these patients should be treated with half the regular dose of aripiprazole normally prescribed.

1.6.5 Quetiapine

Quetiapine is a second-generation or atypical antipsychotic drug (AAD) developed in 1985 by scientists at AstraZeneca Pharmaceuticals and used in the symptomatic treatment of severe mental illness like schizophrenia, psychosis, bipolar I disorder and major depressive disorder (MDD) (**Figure 1.8**).

In patients with bipolar I disorder quetiapine may be used, as a monotherapy or combined with other drugs, to manage acute depressive episodes, mixed manic depressive episodes, and major depression in combination with antidepressant drugs (Shotbolt, Samuel and David, 2010; Maneeton *et al.*, 2016).

Quetiapine is also used off-label for the management of generalized anxiety disorder (GAD), post-traumatic stress disorder (PTSD) and psychosis associated with Parkinson's disease (Maneeton *et al.*, 2016).

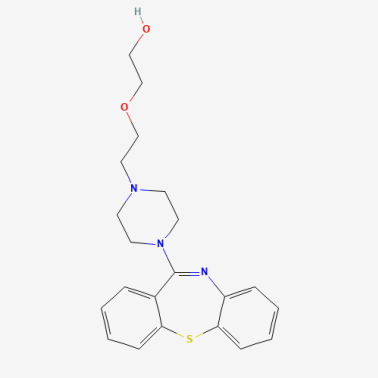

Figure 1.8: Quetiapine structure. A two-dimensional representation of the compound is detailed. From https://pubchem.ncbi.nlm.nih.gov/compound/5002.

1.6.5.1 Pharmacodynamics

The therapeutic effect of quetiapine has been demonstrated in several clinical studies, and is expressed through the improvement of positive and negative symptoms of schizophrenia as well as major depression through its action on various neurotransmitter receptors, such as dopamine and serotonin receptors (Riedel *et al.*, 2007).

Due to an increased death incidence in senior patients taking quetiapine, this antipsychotic medication is not indicated for the treatment of psychosis related to dementia in elderly. Moreover, Quetiapine can cause suicidal thinking or behavior in younger patients and should not be given to

children and adolescents under 10 years of age (Dev and Raniwalla, 2000; Suttajit *et al.*, 2014; Muneer, 2015).

1.6.5.2 Mechanism of action

Though the precise mechanism of action of quetiapine has not been fully comprehended, as with other second-generation antipsychotics, it is most probable that its therapeutic effect is due to its antagonism action on dopamine type 2 (D_2) and serotonin 2A ($5HT_{2A}$) receptors, especially in schizophrenic patients. It has substantial affinity for the limbic system, in particular towards serotonergic ($5HT_{2A}$), histaminergic (H1) and dopaminergic receptors (both D_1 and D_2), moderate affinity to adrenergic receptors alpha-1 and alpha-2 and lower affinity to muscarine receptors M1 type (Riedel *et al.*, 2007). The antagonistic effect on these receptors, however, may be the cause of the development of mild but nonetheless adverse effects from administration of this drug, such as drowsiness anticholinergic effects and orthostatic hypotension (Dev and Raniwalla, 2000; Maneeton *et al.*, 2016). The relatively low incidence of extrapyramidal side effects of quetiapine, on the other hand, is probably due to the antipsychotic characteristics of this class of medications, which have a relatively higher occupancy profile and receptor affinity for the 5HT2A receptor than for the D2 receptor (DeVane and Nemeroff, 2001; Riedel *et al.*, 2007).

1.6.5.3 Metabolism

Quetiapine metabolism is performed mostly in liver. The main metabolic pathways of this drug are oxidation and sulfoxidation (**Figure 1.9**). Quetiapine is metabolized by Cytochrome P450 3A4 to an inactive sulfoxide metabolite and also participates in the metabolism of its active metabolite, N-desalkyl quetiapine. Moreover, 7-hydroxy-N-desalkylquetiapine is a pharmacologically active quetiapine metabolite regulated by CYP2D6. Variation in the concentration of this active

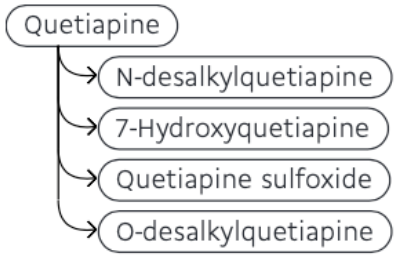

Figure 1.9: Quetiapine metabolism. In the figure the quetiapine metabolism is detailed. The metabolism of quetiapine occurs mainly in the liver. Sulfoxidation and oxidation are the main metabolic pathways of this drug.

metabolite can may be influenced by interindividual differences in CYP2D6 metabolism (Urichuk *et al.*, 2008).

1.6.6 Risperidone

The second-generation antipsychotic risperidone and his primary active metabolite, Paliperidone, are two of the most widely used SGAs (**Figure 1.10**).

Risperidone is prescribed to treat various mental health disorders and conditions, including schizophrenia, psychosis, irritability associated with autistic disorder, bipolar mania and bipolar I disorder. It is also prescribed, sometimes off-label, either in addition to lithium or valproic acid or alone in monotherapy, for the treatment of severe depression, acute mania or mixed manic-depressive episodes associated with bipolar I disorder (Fenton and Scott, 2005; Kemp *et al.*, 2009; Corena-McLeod, 2015).

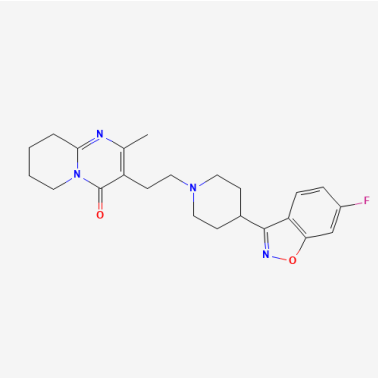

Figure 1.10: Risperidone structure. A two-dimensional representation of the compound is detailed. From https://pubchem.ncbi.nlm.nih.gov/compound/5073.

1.6.6.1 Pharmacodynamics

The primary therapeutic action of risperidone in the treatment of psychosis and various mood disorders is thought to decrease the hyperactivity of central mesolimbic and mesocortical pathways, through the inhibition of dopaminergic and serotonergic receptors activity in the brain, therefore reducing schizophrenia and mood disorders symptoms. (Marder and Meibach, 1994; Fenton and Scott, 2005).

Compared with first-generation antipsychotic drugs, risperidone binds with extreme affinity to serotonergic 5-HT_{2A} receptors, approximately 10-20 times greater than the drug's binding affinity to dopamine D_2 receptors, with which FGAs have a much higher affinity. It is plausible that a

reduction of extrapyramidal symptoms in patients treated with risperidone is due precisely to this inherent moderate affinity for dopamine receptors. In addition, it has less activity on several off-target receptors that may be responsible for some of its undesirable side effects (Marder and Meibach, 1994; Fenton and Scott, 2005; Kemp *et al.*, 2009).

1.6.6.2 Mechanism of action

As with many SGAs, the mechanism of action of risperidone remains largely unknown and not fully understood, however, its therapeutic activity is thought to be related to risperidone's ability to inhibit dopaminergic D_2 receptors and serotonergic 5-HT_{2A} receptors in the brain.

Positive symptoms of schizophrenia and other psychoses, such as hallucinations and delusions, are pharmacologically inhibited transiently by the activity of risperidone, which reduces dopaminergic neurotransmission by binding with low affinity to the D_2 receptors. To obtain an optimal pharmacological effect, the receptor occupancy should be around 60-70%.

The permanent blockade and high occupancy rate of dopaminergic receptors contributes to an increased risk of developing extrapyramidal symptoms and is hence to be prevented. However, this possibility is significantly decreased among risperidone-treated subjects, since the low affinity binding and quick dissociation from the D_2 receptors differentiate risperidone from first-generation antipsychotic medications(Hall *et al.*, 1995; Urichuk *et al.*, 2008).

Negative symptoms of schizophrenia and other psychoses, such as decrease motivation and depression, are caused by an increase in mesocortical serotonergic activity, which is pharmacologically decreased by the action of risperidone, which binds with high affinity to serotonin 5-HT_{2A} receptors (Marder and Meibach, 1994).

Moreover, the decreased incidence of EPS in patients treated with risperidone medication is probably due to a synergic action involving the blockade of both serotoninergic and dopaminergic receptors (5-HT_{2A} and D_2), which probably lead to an increased dopamine release from the frontal cortex instead of from the nigrostriatal tract.

Risperidone is also an antagonist of alpha-1 adrenergic (α1), alpha-2 adrenergic (α2) and histaminergic (H1) receptors (*H1R1*). Although the mechanisms by which the drug acts on these receptors have not been clarified yet, it is known that their blockade results in an improvement in schizophrenia symptoms (Marder and Meibach, 1994).

1.6.6.3 Metabolism

Risperidone is widely metabolized in the liver by cytochrome P450 enzyme (*CYP2D6*) to paliperidone (9-hydroxyrisperidone) its major metabolite, but present another less common metabolite, known as 7-hydroxyrisperidone. CYP3A4 and CYP3A5 enzymes are also capable of metabolizing risperidone to 9-hydroxyresperidone. Paliperidone and risperidone have approximately the same receptor binding affinity, moreover have moderate to strong affinity for P-glycoprotein 1 (*ABCB1*) and also are inhibitors of this protein (Marder and Meibach, 1994; Fang, Bourin and Baker, 1999; Urichuk *et al.*, 2008).

1.6.7 Clozapine

Clozapine is a tricyclic dibenzodiazepine, classified as a second-generation antipsychotic agent, prescribed for the treatment of resistant schizophrenia, to decrease suicidal behavior in schizophrenic patients and for the management of advanced dopamine-mimetic psychosis (**Figure 1.11**).

It displays a distinctive pharmacological profile and binds various different receptors in the central nervous system.

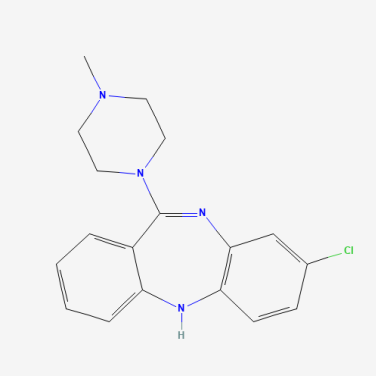

Figure 1.11: Clozapine structure. A two-dimensional representation of the compound is detailed. From https://pubchem.ncbi.nlm.nih.gov/compound/135398737.

1.6.7.1 Pharmacodynamics

Clozapine is a psychotropic agent that belongs to the benzisoxazole derivatives class. Clozapine is a selective high-affinity antagonist for a various number of receptors, with strong binding to 5-HT_{2A} and 5-HT_{2C} receptor subtypes, 1 and 2 adrenergic, and also H1 histaminergic receptors, as well as a selective monoaminergic antagonist. Moreover, clozapine shows weak

antagonism for dopamine type 2 receptors (D_2) and other receptors with low potency (Hall *et al.*, 1995).

The antagonism activity on other receptors, even with low affinities, in addition to the classic dopaminergic and serotoninergic D_2 and 5-HT_{2A} may explain some of the therapeutic effects of clozapine, as well as the development of side effects. Antagonism for M1-5 muscarinic receptors may explain its anticholinergic effects, antagonism at H1 histamine receptors may explain the drowsiness and antagonism at alpha-1 adrenergic receptors may explain the orthostatic hypotension.

Moreover, agranulocytosis and myocarditis are major adverse reaction that can occur after the administration of clozapine antipsychotic. Patients with agranulocytosis, which is a reduction in absolute neutrophil or white blood cell counts usually occurs in the first 3-6 months of treatment, but can also occur after years of therapy, may also be at increased risk of developing unpleasant infections (Ronaldson *et al.*, 2011).

1.6.7.2 Mechanism of action

The pharmacological action of clozapine is due to a synergistic combination of antagonistic effects at D_2 receptors in the mesolimbic pathway and at 5-HT_{2A} receptors in the frontal cortex.

Positive symptoms of psychoses, such as hallucinations and delusions, are alleviated by olanzapine activity due to its antagonistic activity on dopamine D_2 receptors, while negative symptoms, like depression and decrease motivation, are attenuated by serotonergic receptor antagonism on 5-HT_{2A}.

1.6.8 Olanzapine

Olanzapine is a second-generation antipsychotic medication, belonging to the thienobenzodiazepine class, discovered and approved to be marketed in 1996 in the US (**Figure 1.12**).

Chemically very similar to clozapine, olanzapine is used to treat schizophrenia, psychosis and bipolar I disorder. Furthermore, it is administered for the management of psychomotor agitation and delirium associated with schizophrenia and with bipolar I mania.

In combination with lithium or valproate olanzapine is also indicated for the treatment of acute manic or mixed episodes associated with bipolar I disorder, an altered mental condition brought about by periods of extreme mood disturbance, while in combination with fluoxetine is used for the depression associated with bipolar I disorder and treatment-resistant depression in young patients, over 10 years old.

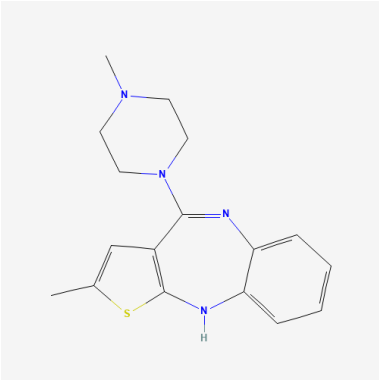

Figure 1.12: Olanzapine structure. A two-dimensional representation of the compound is detailed. From https://pubchem.ncbi.nlm.nih.gov/compound/135398745

1.6.8.1 Pharmacodynamics

As with all second-generation antipsychotics, olanzapine's main effect is dual, involving both serotonin (5-HT_{2A} and 5-HT_{2C}) and dopamine receptors (D_1-D_4) although it has a range of additional receptor affinities for histamine H1 receptor (*HRH1*), alpha1 adrenergic receptors, and muscarinic receptors (*CHRM1- CHRM5*) (Callaghan *et al.*, 1999).

Regarding the positive effect of olanzapine on the dopamine D_2 receptor, it determines a decrease in delusions, hallucinations, disorganized thinking and speech and also disorganized behavior. Olanzapine's higher affinity for the dopamine D_2 receptor than other dopaminergic receptor types is due to its specific mechanism of action. This property therefore significantly reduces the development of undesirable side effects in patients treated with this medication.

Moreover, its antagonism on the serotonin 5-HT_{2A} receptor prevents the onset of negative symptoms related to psychosis such as anhedonia, alogia, anaphectivity, lack of motivation and poor attention (Thomas and Saadabadi, 2021).

Several clinical trials demonstrated significant effectiveness of olanzapine in the treatment of schizophrenia and bipolar disorder especially in adults, while in adolescents it has been shown to be effective in managing acute or mixed manic episodes associated with bipolar disorder.

moreover it has been shown that olanzapine can lead to a significant increase in the management and control of nausea and vomiting (Brunner *et al.*, 2013; Yang *et al.*, 2017).

1.6.8.2 Mechanism of action

Olanzapine action is based through its antagonism activity on different neuronal receptors in the brain, including dopamine D_1 receptors and D_2-like group receptors (D_2, D_3 and D_4), serotonin receptors 5-HT_{2A}, 5-HT_{2c}, 5-HT_3 and 5-HT_6, the alpha-1 adrenergic receptor, the histamine receptor H1 (*HRH1*) and multiple muscarinic receptors (*CHRM1- CHRM5*) (Callaghan *et al.*, 1999).

Although it has affinity with a wide variety of targets, the antagonistic effect of olanzapine towards the dopamine D_2 receptor in the mesolimbic pathway is critical because it blocks dopamine from having action potential at the postsynaptic receptor.

Olanzapine binding to dopamine D_2 receptors is readily dissociable and thus allows some degree of dopamine neurotransmission.

In addition, olanzapine binds on serotonin 5-HT_{2A} receptors in the frontal cortex in the brain in an analogous way to that reported for dopamine D2 receptors, leading to a decrease in adverse effects (Callaghan *et al.*, 1999).

1.6.8.3 Metabolism

Olanzapine is mainly metabolized in the liver, mostly by the activity of the cytochrome P450 and by direct glucuronidation. From the CYP enzyme group, the main metabolic enzymes involved are CYP1A2, CYP2D6 and CYP3A4.

In the context of phase I metabolism, the main circulating olanzapine's metabolites are 10-N-glucuronide and 4'-N-desmethyl olanzapine, which are clinically inactive and formed by CYP1A2 activity. Moreover, CYP2D6 catalyzes the formation of 2-OH olanzapine and flavin-containing monooxygenase (FMO3) is responsible for N-oxide olanzapine. Regarding phase II olanzapine metabolism, UGT1A4 is the main player generating direct forms of olanzapine conjugation (Callaghan *et al.*, 1999; Urichuk *et al.*, 2008).

1.7 CLINICAL PRESENTATION AND OUTCOME

The clinical presentation of NMS is varied and heterogeneous, clinical signs and symptoms are not homogeneous, and often differ from case to case, thus making diagnosis complicated, especially in the initial phase. The fact that the development of NMS is the unexpected and unanticipated result of prior exposure to certain categories of substances or drugs, primarily antipsychotics, make the onset of the syndrome and its clinical features particularly complex (Mann *et al.*, 2003; Oruch *et al.*, 2017).

Although there is variability in the onset of the disorder, and NMS usually occurs through an inexplicable combination of different symptoms: muscle tremor and cramping, hyperthermia, labile blood pressure, and mental status alteration (agitation, anxiety, delirium) and sometimes fulminant coma.

In some cases, NMS may evolve as soon as a few hours after drugs administration. In most cases, however, NMS usually develops over the course of several days, typically up to a maximum of 10 days after the introduction of a drug, and the full clinical picture develops within 48 hours of the first symptoms appearance in more than 90% of the cases. However, the onset of SNM can also occur as short as 4-5 hours and as long as approximately 65 days after the first administration of a medication. In this regard, Caroff and Mann reported, in a review of NMS cases they conducted, that 16% of patients developed signs of the syndrome within one day of drug initiation, 66% within 1 week and 96% within one month, while the least likely condition was that NMS occurred 30 days after drug initiation (in the 4% of cases). On average, the duration of a neuroleptic malignant syndrome episode is approximately 15 days (2 weeks), which can be up to 1 month due to the administration of depot preparations (Caroff and Mann, 1988; Mann *et al.*, 2003; Ananth *et al.*, 2004; Oruch *et al.*, 2017).

1.7.1 Early Signs

The recognition of early or prodromal clinical manifestations of NMS would be useful to facilitate early diagnosis and timely intervention in terminating the syndrome. Typically, NMS develops subtly over days and is preceded by autonomic and neurologic signs that are unresponsive to traditional treatment, but it can also occur abruptly and develop within hours, thereby precluding identification of initial signs.

Early signs that can precede NMS may comprise mental status alterations (reported in 97% of NMS cases) characterized by delirium, catatonia, dullness, or distorted consciousness oscillating from stupor to coma; affected subjects may seem vigilant in appearance, but simultaneously stunned, non-reactive, and mute, coherent with an appearance of catatonic stupor and akinetic mutism. Episodic tachycardia, tachypnea, or hypertension, incontinence, body temperature elevations, vital signs variations and extrapyramidal function changes that do not respond to antiparkinsonian agents are considered initial signs as well (Caroff and Mann, 1988; Mann *et al.*, 2003).

In most cases, generalized muscle stiffness, typically described as "lead pipe" rigidity in its gravest form, is considered a major characteristic of NMS. This hallmark sign may not respond to treatment with antiparkinsonian medications and may be combined in association with other neurological or motor symptoms, including altered muscle tone, tremor, akinesia, myoclonus, dysphagia, dysarthria, sialorrhea and trismus. In addition, these clinical features may also be accompanied by rhabdomyolysis, and changes in mental status announce the onset of the syndrome and are followed later by a cascade of systemic signs of hypermetabolism (Mann *et al.*, 2003; Ware, Feller and Hall, 2018).

Some researchers have proposed that mental status alterations, extrapyramidal and autonomic symptoms usually develop before other signs of NMS, whereas hyperthermia and hypermetabolism develop relatively later, reflecting the culmination of pathophysiological processes. Velamoor and colleagues, finding a predominance of the presentation of four signs and symptoms typical of NMS, proposed in a review a sequential pattern of the presentations of these symptoms. Their study discovered that more than 70% of the time, the precise sequence of clinical signs that occur in NMS patients and define the syndrome is as follows: first, alterations in mental status, secondarily muscle rigidity, then followed by hyperthermia, and finally autonomic dysfunction. Therefore, clinicians should be aware that, in some cases and patients, the temporal sequence with which clinical symptoms and the typical manifestations attributable to the syndrome appear may be crucial for the early diagnosis of NMS and help clinicians to the timely intervention. However, these signs are not unambiguous and exclusive, but more importantly, they do not necessary precede the onset of the syndrome and do not invariably progress to NMS. The diagnosis of NMS should always be considered by clinicians and medical staff if a patient appears to deteriorate neurologically following treatment with antipsychotic medications (Velamoor *et al.*, 1994; Mann *et al.*, 2003; Ware, Feller and Hall, 2018).

1.7.2 Clinical signs and symptoms

NMS patients exhibiting typical syndrome characteristics, usually may present with an adrenergic, hypothalamic, hypodopaminergic, or parkinsonian seizure and/or convulsions, with a manifestation of hypermetabolism and subsequent elevation of oxygen consumption.

According to many clinicians and experts, hyperthermia is the most distinctive feature of the neuroleptic syndrome, distinguishing it from other conditions and clinical onsets due to the intake of antipsychotic drugs, but which may present with similar manifestations such as extrapyramidal, autonomic and neuropsychiatric dysfunction, albeit in various combinations, and usually develops in the final stages of the syndrome, as a retarded manifestation, and does not respond to administration of conventional antipyretic drugs. During the hyperthermic phase, human body temperature can range from 38°C to 40°C, exceeding 38°C in 87%. Hyperpyrexia, which is an increase in body temperature above 40°C, has been found in 40% of cases, although temperatures above the 42°C limit of standard thermometers have also been reported. Among the plausible mechanisms leading to the development of hyperthermia and hyperpyrexia in NMS antipsychotic-induced inhibition of central dopaminergic thermoregulatory pathways, primarily at the hypothalamus level, that mediate warmth dissipation and the augmented temperature production resulting from the effects of antipsychotic on metabolism and skeletal muscle tone, are reported (Caroff and Mann, 1988; Mann *et al.*, 2003; Oruch *et al.*, 2017).

In most cases (approximately 98% of reported NMS cases) extreme sweating occurs along with hyperthermia and/or hyperpyrexia. Interestingly, the same medical conditions that can result from hyperthermia states due to NMS, such as dehydration and electrolyte disequilibrium, which can also increase as a result of excessive sweating, infections, pulmonary embolism, seizures, and rhabdomyolysis, may themselves contribute to the increase in body temperature as a secondary condition, although they are not the primary cause of the initial temperature rise and development of hyperthermia. Extreme hyperthermia and hyperpyrexia can make patients prone to developing medical complications, especially if predisposing elements such as hot weather, excessive exercise, dehydration, or agitation are present, predisposing them to develop heatstroke, cerebellar damage or irreversible brain trauma and multi-organ failure, especially if action is not taken promptly to lower body temperature. In addition, it is important to know that hyperpyrexia can appear as a delayed symptom, even 24 hours after the appearance of the first clinical manifestations, and therefore this can lead, even among clinicians and medical experts, to doubts and confusions in making a certain diagnosis (Addonizio, Susman and Roth, 1987; Caroff and Mann, 1988; Mann *et al.*, 2003; Oruch *et al.*, 2017).

Muscular stiffness, although it is defined as one of the early signs of NMS, along with other manifestations such as hypertonicity, or unresponsiveness to antiparkinsonian medications, in association with hyperthermia may be helpful in differentiating the syndrome from other conditions with similar features. Other parkinsonian-like features may be present, such as tremors, which may be recurrent, widespread, symmetrical, but also sialorrhea, bradykinesia, or other neurological features, which may include dyskinesia, dystonia, dysarthria, dysphagia, chorea, myoclonus, trismus, opisthotonos, but also eye seizures, nystagmus, blepharospasm, and ocular flutter (Mann *et al.*, 2003; Oruch *et al.*, 2017).

In some cases of NMS, oculogyric seizures and choreiform movements have also been noted. These manifestations have been seen mainly after intoxication or induced by the administration of antipsychotics such as chlorpromazine, haloperidol, olanzapine or fluphenazine. However, caution should be exercised by clinicians, as other drugs other than antipsychotics, such as anticonvulsants and levodopa, may cause these side effects. Moreover, oculogyric seizures may also be observed in certain forms of epileptic crisis (Mann *et al.*, 2003; Oruch *et al.*, 2017).

In some cases, patients have recurrent and continuous periods of agitation during NMS, and often this condition requires physical immobilization of the subject or sedation through benzodiazepine treatment. Autonomic nervous system alterations, such as tachycardia, tachypnea, fluctuating blood pressure, excessive sweating, and urinary incontinence, may be observed at any time during the development of the syndrome, but their presence may be a very important alarm bell for promptly recognizing NMS (Caroff and Mann, 1988; Mann *et al.*, 2003).

Doctors should be aware that the substantial variations in clinical manifestations and symptoms that may occur in NMS following administration of atypical antipsychotic agents may diverge from those that occur following the administration of typical (or older generation) antipsychotic medications. Therefore, the variances and dissimilarities that arise in the clinical presentation of the syndrome must be accounted for, and signs and symptoms must be assessed twice before a certain and definitive diagnosis of NMS is pronounced.

1.7.3 Laboratory abnormalities

Although there are no specific laboratory findings used in the diagnosis, clinical laboratory abnormalities are generally described in NMS (**Table 1.5**). Most of them, however, are not specific to the syndrome, but rather arise from a variety of symptoms and complications that may be present

during NMS. Nevertheless, a comprehensive laboratory investigation is essential to complete the clinical picture of patients as much as possible, in order to make a confident and certain diagnosis of NMS and furthermore rule out other causes of hyperthermia.

Biochemical and hematologic and alterations are therefore unavoidable: leukocytosis, and serum CPK elevations, which can occur as a result to hyperkinesia and consequent rhabdomyolysis or myonecrosis, can occur in up to 95% of NMS cases, and sometimes increase up to 2000-fold above normal values. In some cases, increases in these laboratory values occur more modestly, making it more difficult to distinguish specific values associated with NMS from less specific values attributable to other more common manifestations, such as agitation. Myoglobinuria, detectable from urine analysis examination, was identified in almost 70% of NMS cases (Mann *et al.*, 2003; Oruch *et al.*, 2017).

Another alteration that can be detected through laboratory investigations involves an increase in serum aldolase, which along with other enzymes (such as lactic dehydrogenase and transaminases) are released into the circulatory stream from damaged skeletal muscle fiber cells. On the contrary, increases in alkaline phosphatase or hepatic bilirubin were not detected in NMS patients.

This pattern of altered laboratory results is most likely due to severe musculoskeletal tissue damage, which can also lead to muscle necrosis, resulting from severe muscle rigidity, hyperthermia, and hyperpyrexia. This set of symptoms and clinical manifestations, due to NMS, can also result to acute renal failure (Mann *et al.*, 2003).

However, the diagnostic validity of serum enzyme quantification analysis has been debated, because several factors, such as agitation or intramuscular injections, may contribute to the increase in CPK levels. Nevertheless, detection of CPK in serum remains fundamental as an approach to detect the severity and risk of renal failure.

In approximately 50% of NMS patients examined by electroencephalographic (EEG) analysis, a peculiar pattern was detected in which electroencephalographic rhythms appeared to change slowly and unexplainedly. According to a plausible hypothesis, these characteristic EEG findings, consistent with encephalopathy in most cases, might be due to changes in neuronal pathways following dopaminergic receptor blockade (Mann *et al.*, 2003; Oruch *et al.*, 2017).

Metabolic acidosis, hyperkalemia, hypoxia, or nonspecific leukocytosis have been found in other cases of NMS (Sahin *et al.*, 2017). Several cases of patients with decreased platelets, peripheral thrombophlebitis, and pulmonary embolization have also been reported. Moreover,

hyperglycemia and sodium alterations (defined as hyper- and hypo-natremia) have also been described in association with the clinical course of NMS, probably due to electrolyte and fluid dysregulation, whereas some other clinicians and researchers have proposed that diabetes, hyponatremia, or deranged antidiuretic hormone secretion should be considered as predicting symptoms of NMS (Mann *et al.*, 2003; J. R. Strawn, Keck and Caroff, 2007).

Another important result of laboratory analysis that was found during NMS concerns serum iron concentration. Although low levels of iron were not observed in all cases, it has been proposed as a marker related to the condition and level of development of NMS, and furthermore may be indicative of the unspecific consequences of an acute phase reaction.

In addition to decreased iron levels, an increase in serum catecholamines and peripheral levels of monoamines and their metabolites have been reported, and probably are the result to the adrenergic crisis that occurs as a consequence of the NMS (Mann *et al.*, 2003; J. R. Strawn, Keck and Caroff, 2007).

Laboratory abnormalities in Neuroleptic Malignant Syndrome	
Parameters	**Changes**
Atypical enzymes and proteins in plasma	
ALP	Increased
ASAT and ALAT	Increased
CK	Increased in 50%-100% of cases
LDH	Increased
Myoglobin	Myoglobinemia
Serum electrolytes and proteolysis remnants	
Calcium	Hypocalcemia
Magnesium	Hypomanesemia
Phosphate	Hyperphosphatemia
Potassium	Hyperkalemia
Sodium	Hypo or hypernatremia
Urea	Uremia (increased BUN)
Uric acid	Hyperuricemia
Blood Elements	
Blood platelets (thrombocytes)	Thrombocytosis (thrombocytopenia*)
Leukocytes	Leukocytosis (70%–80% of cases)
Urine	
Myoglobin	Myoglobinuria (increased myoglobin)
pH (blood gas analysis)	Decreased (metabolic acidosis)
Protein	Proteinuria (increased protein)

Table 1.5: Laboratory abnormalities. In the table are reported laboratory data expected to be altered in patients with Neuroleptic Malignant Syndrome. Abbreviations: ALP, alkaline phosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; LDH, lactate dehydrogenase. Adapted from Oruch et al., 2017.

1.7.4 Outcome

The outcome of the syndrome can vary widely among cases and patients, ranging from a complete recovery to death in the worst cases of NMS sufferers. The average recovery time is approximately 7-10 days after suspension of pharmacologic treatment, and almost all patients improve within 30 days. However, the duration time of NMS episodes may lengthen and even double in subjects treated with long-acting depot antipsychotics (Ware, Feller and Hall, 2018).

Around the 1980s and 1990s, the mortality rate due to NMS ranged from 11% to about 30% worldwide. In the last past years, however, the mortality has diminished considerably to about 10%, undoubtedly due to the increasing awareness of clinicians in knowing and recognizing promptly the characteristics and the onset of the syndrome, the introduction of newer antipsychotic medications, early diagnosis, rapid drug interruption, and also the initiation of specific treatments and supportive care, such as pharmacological therapies and electroconvulsive therapy (ECT) (Mann *et al.*, 2003; Brian D Berman, 2011; Velamoor, 2017; Ware, Feller and Hall, 2018).

According to the estimation made by some clinicians, it would appear that patients who had a fatal outcome were significantly older or more likely to have received depot medications, some had pre-existing brain pathology or had developed coma, and finally some had developed a higher body temperature as a symptom of NMS. The causes of death in NMS patients may be multiple, in fact it may occur as a result of respiratory or cardiac arrest due to heart failure, arrhythmias or infarction; other causes, sometimes difficult to detect, may be pulmonary emboli or pneumonia aspiration, renal failure due to myoglobinuria or intravascular coagulation (Mann *et al.*, 2003).

Although one might expect that, if the syndrome is recognized and treated promptly, patients will recover completely without consequence, cases of individuals developing morbid sequelae during the course of NMS or even permanently are not excluded (Velamoor, 2017).

According to a study conducted on a population of subjects who had experienced NMS, in 70% of cases patients developed a series of symptoms that followed one another in sequence, at the end of which the subject ended up in coma. In these patients, mental status changes first appeared, which were followed by muscle stiffness, next hyperpyrexia, and lastly dysautonomia, which may comprise nausea and vomiting, diaphoresis, labile blood pressure, and cardiac arrhythmias.

At the respiratory level, on the other hand, sequelae can be observed in 31% and can present in various forms, such as tachypnea, chest wall restriction, moderate or severe respiratory failure

that can result from metabolic acidosis, aspiration pneumonia, aspiration due to impaired swallowing or pulmonary emboli, infection, shock, neuroleptic-induced decrease in chest wall volume or due to necrotization of respiratory muscles following rhabdomyolysis; all of these complications can also result in respiratory arrest and lead to death in the most severe cases (Mann *et al.*, 2003; Oruch *et al.*, 2017).

At the neuromuscular level, lingering lesions and consequences have also been referred in a variety of patients with NMS. Among them, permanent polyneuropathy and dystonia, resulting in debility and sensorimotor decline, while enduring muscle stiffness is one of the most encountered enduring lesions and can be severe enough to cause muscular tissue avulsion, joint dislocation, and limb twitching.

As for enduring cognitive sequelae, they would seem to be not as common. However, in some NMS patients have been found cerebellar ataxias, also defined as chronic cerebellar syndrome, a heterogeneous group of neurodegenerative disorders including, among others, extrapyramidal or motor disorders and speech articulation disorders (dysarthria), and organic brain syndromes (expressed in brain degeneration and/or altered mental status). According to some authors, on the other hand, major complications at the level of the nervous system, such as irreversible damage to the cerebellum or other parts of the brain, may arise if a reduction in extreme hyperthermia does not occur promptly. Regardless, brain deficits have been noted and would appear to be attributable to the complications of the syndrome, in particular hypoxia and severe or persistent hyperthermia, or the degree of its severity (Mann *et al.*, 2003; Ananth *et al.*, 2004).

Moreover, NMS patients could be a higher risk to develop morbidity as a consequence of various manifestations due to the syndrome, such as deep vein thrombosis and pulmonary embolism as a result of bed restraint and dehydration, aspiration pneumonia due to altered mental status combined with difficulty deglutition and disseminated intravascular coagulation (DIC) following rhabdomyolysis. Occasionally, rhabdomyolysis may present or evolve into such a severe form that it consequently causes myoglobinuric renal failure, one of the most common and severe complications of NMS cases; this condition may lead to the need for hemodialysis if renal failure persist. In addition, other medical complications including convulsions, arrhythmias, cardiopulmonary failure, myocardial infarction, and sometimes sepsis, may occur concurrently with or following the development of NMS, thus requiring intensive care observation and medical support in most cases (Mann *et al.*, 2003; Brian D Berman, 2011).

1.8 RECHALLENGE AND RECURRENCE

As previously mentioned, in recent years the mortality rate due to NMS has diminished significantly, partly as a result of increased awareness among physicians and the introduction of new antipsychotic medications, although all antipsychotics potentially may cause NMS at any time during therapy.

Unfortunately, the individuals most at risk for NMS occurrence following antipsychotic administration are also those who require lifelong drug therapy for the treatment of their psychotic disorders. Therefore, therapy with these categories of medications should be reinstated as soon as possible (Mann et al., 2003; Berman, 2011; Pileggi and Cook, 2016).

Rates of recurrence, reported in case reports and literature, can fluctuate and be as high as 30%. According to a report published by Wells and colleagues, out of approximately 40 cases of NMS, the recurrence percentage would appear to range between 13% of cases confirmed, and 37% of cases (defined as probable), furthermore they reported that symptom recurrence seemed to even double when therapy was restored within 5 days after recovery (Wells, Sommi and Crismon, 1988; Mann et al., 2003). In the same year, Susman and Addonizio reported that of 35 patients who had experienced NMS, 42.9% had suffered a recurrence; furthermore, they observed that the risk of NMS relapse increased when pharmacological treatment was reinstituted before 2 weeks after recovery (Susman and Addonizio, 1988). However, NMS case reports usually do not describe follow-up care and repeat therapy (Mann et al., 2003; Pileggi and Cook, 2016).

After the complete resolution of NMS symptoms, followed by a period of drug-free clearance of at least two weeks, reintroduction of previously administered drug treatment may be undertaken. However, clinicians should consider re-administering neuroleptics other than those that caused the syndrome or, indeed, an alternative agent for their patients. In addition, if prolonged antipsychotic therapy is mandatory, it would be decidedly prudent and necessary to start with a low initial dose and choose a drug that already has low affinity for the D2 receptor (like quetiapine) or is a partial dopamine antagonist and agonist (eg. aripiprazole). Although data on the association between antipsychotic drugs potency or dosing and NMS recurrence are inconclusive, gradual administration of low drug doses or low potency may be, at least initially, preferred. Moreover, if NMS occurred after the administration of depot antipsychotics, then a longer drug-free period, of at least 30 days, is necessary for the subject to comply (Mann et al., 2003; Pileggi and Cook, 2016; Velamoor, 2017).

Possible medications to consider as alternatives to antipsychotics, both first- and second-generation, may comprise mood stabilizers (for instance lamotrigine, or lithium) and also benzodiazepines, which should be prescribed and used, however, cautiously (Velamoor, 2017).

However, as described by some case reports, recurrence can occur regardless of the antipsychotic or medication used. Moreover, acute relapse of NMS may be related more to the time between the resolution of the syndrome and re-administration of drug treatment than to the antipsychotic agent itself. In addition, the genetic component of NMS may predispose patients to the risk of developing the condition again, but it appears that other cofactors must still be present because several reports show relapses after remission of NMS. (Wells, Sommi and Crismon, 1988; Pileggi and Cook, 2016).

A wise approach toward medication rechallenge should consider the patient's overall health after recovery from NMS, particularly whether the individual has morbid sequelae, permanent or otherwise, and the complexity of the underlying psychiatric disorder. In some cases, if discontinuation of antipsychotic therapy is likely to create intolerable disturbances in the patient, it may be necessary to restart therapy earlier than is usually expected and recommended, even 5 days after recovery from NMS.

For individuals, however, who have an elevated risk of developing NMS again or who experience sequelae as a result of the syndrome, one possible alternative to rechallenge that should be considered into account involves the use of ECT for the treatment of psychosis. In any case, regardless of the pharmacologic treatment adopted and the length of the drug withdrawal period adopted between resolution of the NMS and rechallenge, a careful supervising and slow titration are necessary in any patient in order to avoid the risk of any relapse (Pileggi and Cook, 2016).

1.9 DIAGNOSTIC CRITERIA

Although differences among NMS clinical cases and patients have been found and reported, and because there are no "gold standard" or features that can be specifically attributed to the syndrome, NMS has been and still is identified according to precise diagnostic criteria (Mann *et al.*, 2003; Tse *et al.*, 2015).

Since the mid 1980s to the present there have been many researchers who have proposed to standardize diagnostic criteria including Levenson's group, Pope's group, Addonizio's group, Caroff and colleagues, Gurrera's group and others, on the basis of the clinical and laboratory signs described in case reports of neuroleptic malignant syndrome (Levenson, 1985; Addonizio, Susman and Roth, 1986; Pope, Keck and McElroy, 1986; Caroff and Mann, 1993; Mann *et al.*, 2003; Gurrera *et al.*, 2011). Although there are obviously differences, most of the criteria proposed by the various groups include comparable principal features.

Levenson suggested the first series of diagnostic criteria for NMS in 1985. According to his interpretation, in order to make a probable diagnosis of NMS, there had to be the presence of all three major signs (fever, muscle rigidity, elevated creatine phosphokinase) or, alternatively, two major and four or six minor ones (tachycardia, irregular blood pressure, tachypnea, altered consciousness, diaphoresis, and leukocytosis) (Levenson, 1985). Levenson's criteria, although very sensitive and inclusive, were less specific due to the possibility of diagnosing the syndrome even in the absence of muscle rigidity or hyperthermia, considered fundamentals criteria for the diagnosis of NMS by most clinicians, and also did not take into account antipsychotic administration as a trigger (Mann *et al.*, 2003; Tse *et al.*, 2015).

Pope and colleagues, on the other hand, suggested that hyperthermia, autonomic dysfunction, and severe extrapyramidal symptoms were to be considered as hallmarks of NMS, whereas altered mental status, elevated creatine phosphokinase (CPK) and leukocytosis could be considered to make the diagnosis retrospectively. Subsequently, the authors increased the temperature threshold from 37.5°C to 38°C, making these criteria more stringent (Pope, Keck and McElroy, 1986; Mann *et al.*, 2003; Tse *et al.*, 2015).

Most clinicians agree that hyperthermia and muscle stiffness should be considered as the main symptoms for the differential diagnosis. In this regard, Addonizio and colleagues also proposed that the concomitant presence of 10 signs and symptoms, including elevated body temperature (> 37.5°C) and extrapyramidal symptoms in addition to various autonomic manifestations, such as

confused behavior and altered clinical laboratory values, in the absence of other pathologies, could be considered for the diagnosis of NMS (Addonizio, Susman and Roth, 1986). The same criteria have been proposed and codified in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV and most recently DSM-V) (American Psychiatric Association, 1994, 2013). The DSM provides very wide parameters for the diagnosis of NMS that are basically those identified by Caroff and colleagues to which some modifications have been made, although they are used very little in clinical research and patient management (Velamoor, 2017).

According to the criteria proposed by Caroff and colleagues, in order to make a certain diagnosis of NMS the presence of muscle stiffness and fever equal to or greater than 38 °C had to be necessarily found in the patient, along with other signs that, although present, are still to be considered less common, less severe and also difficult to distinguish in the pre-NMS results because extremely highly variable (Caroff and Mann, 1993). Caroff's proposed diagnostic criteria placed exposure to drug treatment with antipsychotics as a key element, as well as specifying that the diagnosis of NMS should be considered after other confounding disorders, such as neuropsychiatric, systemic, and drug-induced hypermetabolic disorders, have been excluded. These criteria highlight that NMS is a diagnosis of exclusion and other similar conditions or neuropsychiatric disorders, that may share a similar clinical picture, should be excluded before diagnosing the syndrome (Caroff and Mann, 1993; Mann *et al.*, 2003).

NMS patients that not completely match the inclusion criteria, and in addition patients that have milder forms or a total lack of muscle stiffness and/or hyperthermia are often classified as “atypical” cases of NMS (Tse *et al.*, 2015). Although some adverse effects from antipsychotic medications may present with symptoms overlapping with those of atypical NMS, calling into question the possibility of its existence, Picard and colleagues sustain the diagnostic validity of the existence of atypical NMS, supported also by a diverse case reports published between 1980 and 2000 believing that, in most cases, the doubt in recognizing atypical NMS stems from the difficulty in distinguishing it from typical prodromal or impending NMS (Picard *et al.*, 2008; Tse *et al.*, 2015).

Adityanjee and colleagues, in order to limit the rate of false-positive as much as possible without excluding *a priori* atypical forms of NMS identified more rigorous parameters that distinguish between four typologies of NMS (Adityanjee *et al.*, 1988; Adityanjee, Aderibigbe and Mathews, 1999). According to the authors, only the first two categories (type I and II) should be considered true NMS, whereas the second two categories (type III and IV) could be classified, at most, probable forms of NMS:

1. **Type I**: true or “classic” NMS induced after the exposure to FGAs or other dopamine medication antagonists, such as antiemetics and antiparkinsonian medications. Other substances and causative agents are not included. Extremely rigorous parameters, in accordance with the diagnostic criteria of NMS suggested by Adityanjee and colleagues in 1988, as well as the assessment of symptom severity by measurement with rating scales, are suggested to diagnose the syndrome and reduce the number of possible false positives.

2. **Type II**: it refers to atypical NMS that arise as a result of taking SGAs but should not be considered as a blander form of type I NMS, because it can also have a severe course, leading to a permanent adverse outcome or even death. In this typology, however, the presence of EPS is not considered essential to formulate a diagnosis of NMS.

3. **Type III**: this situation does not satisfy the parameters of type I and/or type II NMS, as the clinical features are not complete, and all the proposed criteria are not fulfilled. It may be induced by exposure to either FGAs or SGAs. It is assumed that this type, probably consists of a prodromal or imminent form of NMS.

4. **Type IV**: the latter form represents various conditions that overlap with classic NMS. However, there is a change in drugs and medications categories involved, in fact, the syndrome occurs following exposure to psychostimulants, as well as administration of dopamine-lowering drugs or discontinuation of dopamine-agonist medications.

Subsequently, in 2011, an international group of various medical specialists, clinicians and researchers with experience in the care and treatment of NMS patients and NMS cases and from different countries and geographic areas of the world, was created with the intent of establishing a globally recognized set of clinical criteria and medical features for making a correct diagnosis of NMS.

Previous medical evidence and diagnostic criteria did not provide methods for assessing the importance of each clinical sign or symptomatic feature, distinguishing only between major and minor items, or assigning equal importance to distinctive and non-distinctive qualities of the syndrome. Moreover, earlier published NMS diagnostic criteria were based on physicians' personal experience or reviews of clinical evaluation published in the literature, so they did not agree when compared to each other (Levenson, 1985; Addonizio, Susman and Roth, 1986; Gurrera, Chang and Romero, 1992).

Therefore, the international expert consensus group (IEC) of medical specialists from different backgrounds participated in a Delphi process to identify commonly accepted clinical criteria for

the diagnosis of NMS. The group members included psychiatrists who have published clinical research articles and case reports on NMS and volunteer consultants, both of whom belong to the Neuroleptic Malignant Syndrome Information Service (NMSIS) Professional Advisory Council, as well as specialists in neurology, anaesthesiology and emergency medicine. The Delphi method is particularly useful when applied to various clinical and health issues, when experimental evidence is deficient or difficult to obtain, in order to develop consensus among clinicians. In fact, with this method, each clinical parameter or sign is assigned a values of prioritization and unequivocal score, allowing unambiguous identification of objective criteria that can lead to a more reliable and specific diagnosis (Gurrera *et al.*, 2011).

The definitive diagnostic criteria and clinical features agreed upon as a result of the unification of the clinical opinions of the members and experts belonging to the IEC are as follows (also summarized in **Table 1.6**):

- recent exposure to dopamine antagonist, or dopamine agonist withdrawal (within 72 h);
- hyperthermia, with temperature greater than 38.0°C (>100.4 °F) measured orally on at least 2 occasions;
- muscular rigidity;
- mental status alterations (reduced or fluctuating level of consciousness);
- creatine kinase elevation (at least 4-fold above the upper limit of normal);
- sympathetic nervous system lability, characterized as at least 2 of the following manifestations:
    - blood pressure elevation (systolic or diastolic ≥ 25% above baseline),
    - blood pressure fluctuation (diastolic change ≥ 20 mmHg or systolic variation ≥ 25 mmHg within 24 hours);
- diaphoresis;
- urinary incontinence;
- hypermetabolism, described as increased heart rate and increased respiratory rate (≥ 25% above baseline and ≥ 50% above baseline, respectively);
- negative workup due to infectious, toxic, metabolic, or neurologic causes (Gurrera *et al.*, 2011, 2017).

By adding together, the priority points assigned by the IEC to each criteria, an accurate diagnosis of NMS can be made through a single cut-off value score greater than or equal to 74 out of a total of 100 points, which permits the correct identification of more than 85% of NMS cases to be correctly recognized from baseline. However, adoption of these broadly approved diagnostic criteria, although they need to be further tested in patients more medically and demographically heterogeneous before being regularly applied in medical care and practice, may result as an improvement for both research and medical management of cases (Gurrera *et al.*, 2011, 2017; Velamoor, 2017).

Recently, the diagnostic criteria approved by the International Expert Consensus (IEC) have been validated in the clinical patients setting using the DSM-IV-TR diagnostic approach as the "gold standard", since no definite and unique biological marker for NMS is currently available. In addition, the diagnostic criteria validated by the International Expert Consensus (IEC) were also included in the general description of NMS among the clinical features reported in the latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (Gurrera *et al.*, 2011, 2017; American Psychiatric Association, 2013) (**Table 1.3**). It is common hope that this will be a first real step toward the progression of research in the field, as well as the clinical recognition and treatment of patients with NMS.

Levenson (1985)	Pope *et al.* (1986)	Addonizio *et al.* (1986)	Caroff and Mann (1993)	DSM-IV (1994)	Adityanjee, Mathews and Aderibigbe (1999)	DSM-V (2013)	Gurrera *et al.*, (2011, 2017)	
All three major, or two major and four minor criteria suggest a high probability of NMS, if supported by clinical history (for example, not indicative of MH). **Major Criteria:** 1. Fever 2. Rigidity 3. Elevated CPK **Minor Criteria:** 1. Altered consciousness 2. Tachycardia 3. Abnormal blood pressure 4. Tachypnoea 5. Diaphoresis 6. Leukocytos	**Prospective diagnoses** (all three items are required for a definite diagnosis): 1. Hyperthermia (oral temperature >37.5°C in the abscence of another etiology) 2. EPS with at least two of the following: lead-pipe muscular rigidity, cogwheeling, sialorrhea, oculogyric crisis, retrocollis, opisthotonos, trismus, dysphagia, choreiform movements, dyskinetic movements, festinating gait, flexor-extensor posturing 3. Autonomic dysfunction with two or more of the following: hypertension (>20mmHg rise in diastolic above baseline), tachycardia (>30 beats/min above baseline), tachypnoea (>25 respirations/min), prominent diaphoresis, incontinence **Retrospectively, if one of the criteria above is absent, a probable diagnosis is still permitted if the patient displays one the following):** 1. Clouded consciousness 2. Leukocytosis (>15,000 WBC/mm^3) and CPK >300 U/mL	1. Hyperthermia (at least 37,5°C in the absence of other systemic illness) 2. Rigidity 3. Tremor 4. Blood pressure elevation (>140mmHg systolic, >90mmHg diastolic, or both) 5. Tachycardia (at least 100 beats/min) 6. Diaphoresis 7. Incontinence 8. Elevated CPK 9. Leukocytosis 10. Confusion The occurrence of 5 out of 10 symptoms in the same 48 h period is used o identify an episode. The abscence of fever and EPS preclude the diagnosis of NMS.	1. Treatment with neuroleptics within 7 days of onset (2-4 weeks for depot neuroleptics) 2. Hypertehrmia (≥38°C) 3. Muscle rigidity 4 Five of the following: a. Change in mental status; b. Tachycardia; c. Hypertension or hypotension; tachypnoea or hypoxia; e. Diaphoresis or sialorrhea; f. Tremor; g. Incontinence; h. CPK elevation or myoglobinuria; i. Leukocytosis; l. Metabolic acidosis 5. Exclusion of other drug-induced, systemic, or neuropsychiatric illness All five criteria above are required for a diagnosis.	**A**. Development of severe muscle rigidity and elevated temperature associated with the use of neuroleptic medication. **B**. Two (or more) of the following: 1)Diaphoresis 2) Dysphagia 3) Tremor 4) Incontinence 5) Changes in level of consciousness 6) Mutism 7) Tachycardia 8) Elevated or labile blood pressure 9) Leukocytosis 10) Laboratory evidence of muscle injury (eg, elevated CPK) **C**. The symptoms in criteria A and B are not due to another substance or a neurological or other general medical condition **D**. The symptoms in criteria A and B are not better accounted for by a mental disorder	1) Altered sensorium (confusion, clouding of consciousness, mutism, stupor or coma) Rating of severity should be done by at least two independent observers. Non-specific changes in mental status should not be considered. 2) EPS (muscle rigidity, dysphagia or dystonia) 3) Hyperthermia (> 38.5°C measured orally and sustained for at least 48h) in absence of other medical conditions that could explain the elevation in temperature. 4) Autonomic dysfunction (at least 2 of the following) i) Tachycardia (pulse more than 100/min) ii) Tachypnoea (respiration more than 25/min) iii) Blood pressure fluctuations (at least a change of 30 mmHg in systolic pressure or 15 mmHg in diastolic pressure) iv) Excessive sweating (diaphoresis) v) New onset incontinence 5) Relationship of onset of symptoms with exposure event defined by any one of the following i) p.o. ingestion or parenteral administration (dose increase, dose decrease, discontinuation) of an antipsychotic drug (typical or atypical), a dopamine depleter (e.g. tetrabenazine) dopamine blocker (e.g. metoclopramide) or a psycho- stimulant drug (e.g. cocaine) during the previous 2 weeks ii) Withdrawal of antiparkinsonian (e.g. amantadine) or anticholinergic drug during previous 1 week iii) i.m. administration of a long-acting depot antipsychotic medication during the previous 8 weeks 6) Exclusion of any other medical condition 7) Supportive features (any two of the following) (i) Elevations in serum CPK levels (ii) Leukocytosis (iii) Low serum iron levels (iv) Elevation of liver enzymes (v) Myoglobinuria **Type I NMS diagnosis:** Criteria (1)–(6) must be present **Type II NMS diagnosis:** Criteria numbers (1), (3) and (4), (5), (6) and any one item from criteria number (7) must be present for the diagnosis. Criteria number (2) is not necessary for making diagnosis. Using standardized rating scales to measure symptoms severity is recommended.	**1**. Hyperthermia (oral temperature >38.0°C on at least 2 occasions) **2**. Rigidity **3**. CPK >4-times the upper limit **4**. Changes in mental status (delirium, altered consciousness) **5**. Autonomic activation, including: tachycardia (>25% above baseline), diaphoresis, blood pressure elevation (systolic or diastolic ≥25% above baseline), or fluctuation (≥20 mmHg diastolic change or ≥25mmHg systolic change), urinary incontinence, pallor, tachypnea (>50% above baseline)	**Criterion**	**Score**
							1. Exposure to dopamine antagonist, or dopamine agonist withdrawal, within past 72 hours	20
							2. Hyperthermia (> 100.4°F or > 38.0°C on at least 2 occasions, measured orally)	18
							3. Rigidity	17
							4. Mental status alteration (reduced or fluctuating level of consciousness)	13
							5. CPK (at least 4 times the upper limit of normal)	10
							6. Sympathetic nervous system lability, defined as at least 2 of the following: a. Blood pressure elevation (systolic or diastolic ≥ 25% above baseline) b. Blood pressure fluctuation (≥ 20 mm Hg diastolic change or ≥ 25 mm Hg systolic change within 24 hours) c. Diaphoresis d. Urinary incontinence	10 5
							7. Hypermetabolism, defined as heart-rate increase (≥ 25% above baseline) AND respiratory-rate increase (≥ 50% above baseline)	7
							8. Negative work-up for infectious, toxic, metabolic, or neurologic causes	

Table 1.6: Comparison of Diagnostic Criteria for Neuroleptic Malignant Syndrome. In the table are reported the main sets of NMS Diagnostic Criteria, published from 1985 to the present days. Abbreviations: DSM, Diagnostic and Statistical Manual; CPK, creatinine phosphokinase; EPS, extrapyramidal symptoms; WBC, white

1.10 DIFFERENTIAL DIAGNOSIS

From the 1980s to the present day, many scientists, researchers, and clinicians have tried to identify common criteria that would help diagnose NMS quickly and accurately, but despite their differences, they all believe that NMS is a diagnosis of exclusion. A multitude of medical and neurological conditions are found to be characterized by clinical signs and symptoms similar to NMS, therefore it is necessary to rule them out with certainty before making a firm diagnosis, especially for disorders that present with hyperthermia and/or muscle stiffness, mental status change, autonomic dysfunction or conditions that may mimic NMS, especially at the neurological level (Mann *et al.*, 2003; J. Strawn, Keck and Caroff, 2007; Velamoor, 2017).

When performing the differential diagnosis, particular attention should be considered when evaluating heatstroke, lithium intoxication, toxic encephalopathies, lethal catatonia, brain abscess, central nervous system infections, such as viral encephalitis and meningitis, which can be difficult to distinguish from NMS and easily lead to misdiagnosis (J. Strawn, Keck and Caroff, 2007; Brian D Berman, 2011; Velamoor, 2017; Simon, Hashmi and Callahan, 2021).

Caroff and Mann realized that the risk of developing serious drug-induced extrapyramidal adverse reactions, including NMS, appeared to be increased in HIV patients or subjects infected with other viruses that can affect brain system and functions. In addition, localized neurologic signs, headaches, lesions regarding the central nervous system such as brainstem and midbrain structures, meningeal signs and cases of status epilepticus may mimic NMS and should be considered in the differential diagnosis (**Table 1.7**) (Caroff and Mann, 1993; J. Strawn, Keck and Caroff, 2007).

Furthermore, different medications may cause symptoms or adverse reaction that can be confused with those of NMS. In fact, in addition to antipsychotic medications, other categories of dopamine antagonists (such as amoxapine, metoclopramide and prochlorperazine) have also been reported to cause the syndrome. In addition, abrupt discontinuation of dopaminergic agents, such as levodopa and amantadine or the GABA-ergic drug baclofen, can also precipitate a similar NMS-reaction. Parkinson's disease patients may develop NMS-like parkinsonian symptoms, such as rigidity and hyperthermia, also called Parkinsonism Hyperthermia Syndrome, due to sudden interruption of treatment with dopamine agonists drugs used specifically to treat Parkinson’s disease. According to Caroff and colleagues, these symptoms are similar to those due to inhibition of dopaminergic function by antipsychotic drugs (Mizuno *et al.*, 2003; J. Strawn, Keck and Caroff, 2007; Velamoor, 2017).

Differential Diagnosis of Neuroleptic Malignant Syndrome
CENTRAL NERVOUS SYSTEM DISORDERS
Infectious
Meningitis or encephalitis
Brain abscess
Sepsis
Human immunodeficiency virus
Structural patology
Seizures
Tumors
Neuropsychiatric
Malingant Catatonia
Delirium
Nonconvulsive status epilepticus
SYSTEMIC DISORDERS
Environmental
Heatstroke
Pharmacological
Malignant hypertermia
Serotonine syndrome
Extrapyramidal side effects
Anticholinergic delirium
Drug-drug interaction
Anticholinergic delirium
Drug withdrawal (Alcohol, Benziodiazepine, Baclofen, etc)
Toxic
Lithium
Salicylates
Heavy metals
Substances of abuse (cocaine, amphetamine, MDMA, etc)
Endocrine and Metabolic
Thyrotoxicosis
Pheochromocytoma
Acute renal failure
Rhabdomyolysis

Table 1.7: Differential Diagnosis of Neuroleptic Malignant Syndrome. In the table are reported several unrelated disorders and conditions as necessary elements to be differentially diagnosed with Neuroleptic Malignant Syndrome. Adapted from (J. R. Strawn, Keck and Caroff, 2007; Brian D Berman, 2011; Pileggi and Cook, 2016).

1.10.1 Central Nervous System Disorders

A thorough analysis of the Neuroleptic Malignant Syndrome Information Service database showed that the most frequent conditions resembling NMS were infections, catatonic symptoms, brain structural disorders, extrapyramidal symptoms, autoimmune disorders and agitated delirium, and most of them are not even due to drug exposure. Therefore, the differential diagnosis of NMS can be separated into CNS disorders and systemic disorders (Mann *et al.*, 2003).

1.10.1.1 Infections

As mentioned before, central nervous system infections, such as meningitis and viral encephalitis, can be complex to differentiate from NMS and easily lead to misdiagnosis, especially when the occurring symptoms are behavioral. Knowing the patient's medical history, as well as prior viral illness, seizures, headache, focal or meningeal signs, and performing neuroimaging examination or CSF screening may aid in diagnosis a viral etiology and thus avoid retardation in treatment of the infection and possible recovery. Patients with HIV immunodeficiency or infected with other viruses that target the nervous or immune systems, may also have an increased risk of developing NMS when treated with antipsychotic medications. In these cases, discriminating between drug-induced effects and viral pathology may be difficult, nevertheless interruption of medication therapy is recommended regardless (Caroff and Mann, 1993; Brian D Berman, 2011).

1.10.1.2 Structural pathologies

Brain injuries that may occur as a result of various types of traumas, strokes, aneurysms, tumor removal, or abscesses, may give rise to symptoms overlapping with NMS and should be evaluated and excluded by specific neurologic examination and brain imaging. Specifically, injuries to certain brain districts, periventricular nuclei of the hypothalamus, or brainstem areas can produce akinetic mutism similar to NMS, probably as a result of damage to dopaminergic pathways. Conversely, through brain imaging and post-mortem examination of cerebral tissues from patients who died of NMS, no consistent or specifically identified patterns of brain disease were found (Caroff and Mann, 1993; Mann *et al.*, 2003).

1.10.1.3 Seizures

Seizures are uncommonly reported in NMS and should prompt investigation of other factors such as structural brain injuries or metabolic causes. Although infrequently cases of nonconvulsive status epilepticus may appear to resemble the syndrome, some elements may be confounding, especially considering that neuroleptics may lower the seizure threshold, and body temperature and CPK increases may appear after seizure activity (Mann *et al.*, 2003).

1.10.1.4 Malignant Catatonia

Catatonia is a life-threatening disorder characterized by febrile states, akinesia, psychomotor deficits, behavioral abnormalities and altered mental status that can depend on both physical and mental illness. It can occur as a result of either various important medical conditions (tumors, encephalitis, brain injuries) and mental disorders (among which the most represented are schizophrenia, depressive disorder or bipolar disorder) and can present with a highly variable set of symptoms that may present mildly or progressively worsen to potentially lethal (Mann *et al.*, 1986; Brian D Berman, 2011).

In psychiatry literature, one of the most controversial issues is related to discriminating between malignant catatonia (MC) and NMS, as both share similar symptoms such as rigidity, increased body temperature, autonomic dysfunction, laboratory anomalies and reaction to treatment with both electroconvulsive therapy (ECT) and benzodiazepines. For more than a hundred years has been well described the potentially fatal development of catatonic and manic conditions in psychotic disorders, in which uncontrolled hyperactivity can precipitate to exhaustion, hyperthermia, astonishment and, in severe cases, even death. Moreover, in some circumstances, the course of the condition can occur with muscle stiffness that cannot be distinguished from that of NMS (Mann *et al.*, 2003).

Castillo and colleagues attempted to identify and describe a clinical distinction between NMS and MC, pointing out that not only the malignant catatonia or “lethal catatonia” have been described before the psychiatric pharmacological advent, but also that both disorders demonstrate diversity in onset, clinical signs and symptoms, and even outcome (Castillo, Rubin and Holsboer-Trachsler, 1989). According to Mann and colleagues, the two conditions would be clinically indistinguishable in more than 20% of cases, a theory also supported by a study they conducted on a total of nearly 300 patients (Mann *et al.*, 1986; J. Strawn, Keck and Caroff, 2007).

Although it may be arduous to distinguish the two syndromes from each other, the incidence of MC may be diminished if its main clinical characteristics are taken into account, in fact the motor features in catatonia are usually preceded by developmental alteration comprising ambivalent attitude, composure, apathetic behavior, excessive negativism, and psychotic alteration, whereas differences between MC and NMS are more difficult to identify in patients treated with antipsychotic medications. Hence, some researchers reputate neuroleptic malignant syndrome as an alternative drug-induced form of malignant catatonia, but the issue remains remarkably unresolved (Brian D Berman, 2011).

Generally, in NMS cases, symptoms should improve or resolve in one or two weeks, whereas in malignant catatonia, antipsychotic medications would appear to be inefficient and may further impair thermoregulation. Therefore, in both cases the suspension of pharmacological treatment would seem to be the most indicated strategy. Indeed, some studies indicate ECT as the most effective treatment in malignant catatonia (Mann *et al.*, 1990, 2003).

1.10.1 Systemic disorders

1.10.1.1 Heatstroke

Heatstroke is a systemic disorder characterized with hyperthermia, excessive sweating, hypotension, tachycardia, tachypnea, acidosis, confusion and rhabdomyolysis, which can degenerate to renal failure and lead, in already severe cases, even to death. During warmer weather or particularly hot and humid days, agitated patients are at greater risk for exertional heatstroke, whereas the "classic" form is not exertion-related and is characterized by respiratory alkalosis and anhydrous (Mann *et al.*, 2003; J. R. Strawn, Keck and Caroff, 2007).

The differential diagnosis of heatstroke from NMS can be difficult in a psychiatric patient treated with antipsychotic medications. In addition, the same administration of antipsychotics raises the risk of heatstroke by predisposing patients to develop what is known as antipsychotic drug-related heatstroke (ADRHS), although most cases of antipsychotics-associated heatstroke more closely resemble "classic heatstroke" than "exertional heatstroke"(Mann *et al.*, 2003).

Differently from NMS, neither of the two different types of heatstroke is typically associated with muscle stiffness. However, muscle flaccidity and dry skin have usually been observed in heatstroke patients in addition to a prior history of exertion or exposure to high environmental temperatures (J. R. Strawn, Keck and Caroff, 2007).

It is important for clinicians to be aware that, during warmer seasons, administration of antipsychotic medications may increase the risk of heatstroke in treated patients, although NMS can occur regardless of ambient temperature. Another aspect not to be underestimated concerns anticholinergic drugs, in fact the co-administration of them will lead to important impairment of thermoregulation. In this regard, it has been seen that heatstroke is more frequently associated with the administration of low-potency antipsychotics, which possess anticholinergics, than the development of NMS, which is more frequently associated with high-potency medications (Mann *et al.*, 2003).

1.10.1.2 Malignant Hyperthermia

Malignant hyperthermia (MH) is a drug-induced hypermetabolic syndrome that can occur in patients following the administration of anesthetic gases and succinylcholine during general anesthesia. Although the clinical presentation and symptomatology of the two syndromes may appear indistinguishable, actuality, the studies conducted on both MH and NMS suggest that the mechanisms underlying these two adverse events are distinct, in fact, the history of exposure to depolarizing muscle relaxants, most frequently succinylcholine, or volatile anesthetics makes the differentiation between the two syndromes fairly easier in almost all cases (Mann *et al.*, 2003; Simon, Hashmi and Callahan, 2021).

The susceptibility to the development of both syndromes in the same patient is not demonstrated. NMS in fact, unlike MH, does not occur during total anesthesia because the anesthetics and muscle relaxants employed during operation presumably inhibit the central pathways underlying its onset. Therefore, both syndromes are successfully treated pharmacologically with dantrolene, however, only NMS responds to both the dopamine agonist drug and electroconvulsive therapy (ECT) approaches (Mann *et al.*, 2003).

1.10.1.3 Serotonin Syndrome

Serotonin syndrome (SS) is the uncommon but potentially life-threatening adverse reaction most likely to be misdiagnosed as NMS, particularly in its more serious presentations, and deserves special consideration regarding differential diagnosis. Serotonin syndrome is related to serotonin excess and encompasses a variety of autonomic, developmental, and neuromuscular signs that may occur subsequent to serotonergic medications administration, with specific clinical

features such as hyperkinesis, hyperreflexia, myoclonus and incoherent speech. Neuroleptic malignant syndrome, instead, is characterized by bradykinesia, hyperpyrexia, lead pipe rigidity or other extrapyramidal features, autonomic instability, and mental status change (Mann *et al.*, 2003; Margetić and Aukst-Margetić, 2010; Perry and Wilborn, 2012; Tse *et al.*, 2015).

Recently, several cases of SS that exhibited symptoms commonly associated with NMS have been described in the literature. This seems to suggest that the two syndromes, in addition to having common features, also share a certain pattern of symptoms, especially when the triggering agents, or combinations thereof, have both serotonergic and dopamine-antagonist activity (Perry and Wilborn, 2012; Mazhar *et al.*, 2016; Werneke *et al.*, 2016; Scotton *et al.*, 2019).

Because of the similarity and overlap in symptoms and clinical presentation, it is not surprising that SS is confused with NMS, however, a more accurate assessment of the patients' clinical picture may help differentiate the two adverse reactions. However, in most circumstances, SS differs from NMS in onset, clinical manifestation, and outcomes. SS usually arises rapidly, within 24 hours after the introduction of medications or therapeutic changes that affect serotonin levels, but it most often results from combined drug use or overdose, thus much more promptly than NMS. SS patients typically present with agitated delirium, motor agitation and myoclonus (a common and defining feature of the syndrome), gastrointestinal symptoms such as diarrhea, nausea, and vomiting, the absence of leukocytosis, and elevated CPK, whereas NMS patients are usually silent and unresponsive and usually do not present with gastrointestinal symptoms. In addition, SS is more clearly distinguished from NMS by its causative agent, generally specific serotonin reuptake inhibitors (SSRI), and the hyperthermia and muscle stiffness that result during the syndrome are typically less severe than in NMS (Margetić and Aukst-Margetić, 2010; Brian D Berman, 2011; Pileggi and Cook, 2016; Simon, Hashmi and Callahan, 2021).

Overlapping clinical features and symptoms confound the differential diagnosis, whereas clinical laboratory findings are illuminating, therefore, it is highly suggested to pay special attention to laboratory findings when making a differential diagnosis of NMS and SS. However, when SS and NMS are difficult to distinguish, the administration of benzodiazepines seems to be the most appropriate and safest treatment strategy (Rusyniak and Sprague, 2005; Perry and Wilborn, 2012).

1.10.1.4 Extrapyramidal Symptoms with Fever

In patients who have developed catatonia following treatment with neuroleptic drugs or in patients with parkinsonism, intercurrent fever caused by metabolic disorders or infections has been reported. Being able to distinguish between benign extrapyramidal symptoms due to medication intake, and concomitant increases in body temperature due to medical illness, and neuroleptic malignant syndrome, can be a challenge for clinicians. However, excluding common causes of fever in these patients before proceeding with a diagnosis of NMS is paramount (Caroff and Mann, 1993; Mann *et al.*, 2003).

In many cases, before a definite diagnosis of NMS is made, extensive laboratory and medical tests are conducted to exclude ongoing infectious processes. In addition to hyperthermia and other hallmarks attributable to NMS, antipsychotic drug administration was also associated with extrapyramidal symptoms, CPK elevation, and myonecrosis. The mechanisms underlying the occurrence of these extrapyramidal effects associated with hyperthermia have never been adequately elucidated, as well as whether they are independent of NMS or reflect the same mechanisms remains to be determined (Mann *et al.*, 2003).

1.10.1.5 Toxins and Drugs

Hyperthermia has been reported following exposure to phenolic compounds and, concomitantly with muscle rigidity, also following carbon monoxide poisoning. Other toxic substances to be considered for differential diagnosis include heavy metals (lead, arsenic) iron salts, fluoride, salicylates, strychnine, methylphenyltetrahydropyridine (MPTP). Furthermore, infections caused by tetanus or staphylococcal toxins should also be excluded through diagnosis (Caroff and Mann, 1993; Mann *et al.*, 2003).

Although central anticholinergic syndrome, which can occur after taking anticholinergic medications, can have a similar course to NMS presenting with confusion and high body temperature, in actuality typical signs of this disorder include dry skin and absence of rigidity, in opposition to the diaphoresis and muscle stiffness that are typical of NMS. In fact, anticholinergic drugs have the property to increase dopaminergic neurotransmission, but show a moderate ability to cause muscle stiffness or hyperthermia. Regardless, clinicians should be careful and take it seriously for differential diagnosis (Mann *et al.*, 2003; Tse *et al.*, 2015; Jamshidi and Dawson, 2019).

NMS-like states and hyperthermia have also often been reported after the administration of prescription drugs, other than neuroleptics or antipsychotics, or after the intake of substances of abuse. Among these, different types of amphetamines, cocaine, psychedelic drugs, and other stimulant substances can lead to rhabdomyolysis and hyperthermia as a result of the agitation caused by abuse of these substances and, in severe cases, even lead to death; in addition, reduced dissipation of body heat due to vasoconstriction can also lead to convulsions or muscle rigidity. Ecstasy or 3,4-methylenedioxymethamphetamine (MDMA), has been associated with hyperthermic states and may cause NMS-like or serotonin syndrome (Kosten and Kleber, 1987; Daras *et al.*, 1995; Demirkiran, Jankovic and Dean, 1996; Wetli, Mash and Karch, 1996; Mann *et al.*, 2003).

Withdrawal from alcohol or sedative-hypnotics can result in altered mental status and behavioral changes, autonomic dysfunction, and increased temperature with excessive sweating, and therefore both can be misdiagnosed with NMS; moreover, rhabdomyolysis may be present in alcoholic subjects (Caroff and Mann, 1993; Mann *et al.*, 2003). The risk of NMS may increase during withdrawal, and, in addition, the differential diagnosis may be very complex if neuroleptic drugs are administered concomitantly, which may also lower the seizure threshold. Therefore, these categories of drugs should be administered with caution and circumspection in these categories of patients (Mann *et al.*, 2003). In addition to alcohol withdrawal, abrupt discontinuation of CNS depressants such as benzodiazepines or barbiturates can also trigger confusion, hallucinations, tremors, hypertension and tachycardia, however, hyperthermia is not usually among the reported symptoms (Francescangeli *et al.*, 2019).

Antidopaminergic drugs and some CNS-acting drugs lower dopaminergic activity and can trigger hyperthermia. Analogously, abrupt discontinuation of drugs that act as dopaminergic agonists, such as amantadine, levodopa, and others used in the treatment of Parkinson's disease and parkinsonian states, is known to give rise to manifestations similar to NMS. However, these adverse drug reactions not due to antipsychotic medications have in common the acute reduction of brain dopaminergic activity, which thus underlies the association between the dopamine antagonistic features of antipsychotic medications and NMS clinical signs (Mann *et al.*, 2003; Mizuno *et al.*, 2003).

Some case reports and clinical evidence suggest that administration of lithium therapies in combination with antipsychotic medications, for example in individuals with bipolar disorder, may increase the risk of extrapyramidal side effects or NMS. Lithium alone, on the contrary, is hardly likely to induce hyperthermia or rigidity. In addition, administration of lithium concurrently with

the occurrence of episodes of NMS may predispose the patient to develop dehydration, renal failure, and even lithium intoxication, resulting in an increased risk of brain damage and toxic encephalopathy (Ryu *et al.*, 2020).

1.11 TREATMENT AND MANAGEMENT

The primary treatments for NMS are focused on prompt recognition of the syndrome, reduction of risk factors, discontinuation of suspected causative drug therapy (antipsychotics or other medications), and immediate initiation of supportive medical care, and detecting and managing complications. If the syndrome arose as a result of abrupt discontinuation of dopamine-agonist medications, however, then they should be reintroduced as soon as possible (Pileggi and Cook, 2016; Velamoor, 2017; Sienaert, van Harten and Rhebergen, 2019).

The treatments that are initially applied are aimed at restoring normal vital functions as much as possible by reducing high body temperature, restoring body fluids, and supporting respiratory, renal and cardiac functions. Furthermore, meticulous surveillance for complications, particularly aspiration pneumonia, thromboembolism, cardiorespiratory and renal failure, is fundamental. (Caroff and Mann, 1993; Mann *et al.*, 2003; Velamoor, 2017).

Because the risk of developing NMS is known to increase with determined variables, comprising the administration of high potency drugs, dehydration, parenteral administration, polypharmacy, comorbidities, extreme carefulness should be exerted in both management and prevention (Pileggi and Cook, 2016; Velamoor, 2017).

1.11.1 Initial non-pharmacological treatment

The clinical strategy adopted as soon as there is suspicion of possible NMS is to discontinue the suspected drug compound, usually antipsychotic medication, or other potential offending agents. This approach is taken even without a definite diagnosis of NMS or with an atypical presentation of the syndrome. However, if the syndrome has occurred as a result of the abrupt discontinuation of a dopaminergic drug, then this drug should be reintroduced as quickly as possible (Brian D Berman, 2011; Tse *et al.*, 2015; Pileggi and Cook, 2016).

1.11.2 Supportive treatment

The following stage in the management of NMS involves supportive medical treatment. Attempts should also be taken to control and modify, if possible, any environmental conditions and behaviors that may be predispose to the progression or worsen of NMS, such as monitoring

and/or reducing room temperature if it is too high, allowing better dissipation of body temperature. Rehydration through significant fluid administration and reestablishment of electrolyte levels are necessary in most cases, especially if CPK levels are very high with the risk of kidney damage, as patients with NMS typically develop dehydration, even following hyperthermia, and thus susceptible to electrolyte imbalances. In this regard, physical measures are also sometimes applied to control the elevated temperature, although not systematically. These measures, which usually involve the application of cooling blankets and the treatment of hyperthermia with ice packs, can both be used to reduce fever, and have the advantage of both being very low-risk and low-cost and procedures, although they are often considered less effective (Brian D Berman, 2011; Tse *et al.*, 2015; Pileggi and Cook, 2016).

Maintaining a slightly alkalotic pH and correcting eventually metabolic abnormalities, in some cases, may be helpful in preventing rhabdomyolysis and acute kidney injury, which may worsen in renal failure. Other complications may include pneumonia, respiratory failure, and sepsis (Brian D Berman, 2011; Pileggi and Cook, 2016).

Finally, it is important to monitor for aspiration pneumonia, a sign of NMS that can be exacerbated by changes in patient consciousness states in conjunction with an impaired swallowing reflex, which may be associated with an increased incidence of mortality. To avoid this problem, one measure that is often taken is to position the patient neither lying nor sitting, but with torso inclination at 45 degrees (defined as semi-recumbent position) , significantly reducing the risk of aspiration pneumonia (Tse *et al.*, 2015).

However, in more serious cases of severe stiffness or persistent hyperthermia, where supportive care is not effective or sufficient, it is necessary to proceed with interventions involving the administration of specific pharmacological therapy to resolve the symptoms (Pileggi and Cook, 2016).

1.11.3 Pharmacological treatment

Although a wide variety of medications have been used over the years for the treatment of NMS, currently the most widely used and most convincing pharmacologic treatments for resolution of the syndrome have relied on the use of dantrolene sodium, bromocriptine, amantadine and benzodiazepines (Tse *et al.*, 2015; Pileggi and Cook, 2016).

1.11.3.1 Dantrolene

Dantrolene sodium is a hydantoin derivate and the medication of choice used to treat the management of fulminant musculoskeletal hypermetabolism leading to malignant hyperthermia crisis and usually used for the management of NMS, due to its property of relaxing skeletal muscles by disrupting excitation-contraction coupling by inhibiting calcium outflow from the sarcoplasmic reticulum, consequently decreases intracellular calcium availability and also inhibiting heat production in muscle (Mann *et al.*, 2003; Tse *et al.*, 2015; Pileggi and Cook, 2016).

Although its action is misunderstood, dantrolene has been efficaciously employed in the management of MH triggered following anesthesia and other hypermetabolic disorders because it may be effective in diminishing endogenous warmth production independently of etiopathology (Mann *et al.*, 2003).

Adverse reactions due to dantrolene administration have been reported in rare cases, and do not usually occur when the drug is used for short treatments. Instead, cases of hepatitis, which would appear to be the most widely adverse effect, have been reported but only in association with prolonged dantrolene administration (Pileggi and Cook, 2016).

According to Reulbach and colleagues, dantrolene would be effective in the resolution of NMS, even used alone as monotherapy, but would appear to be associated with increased mortality. However, in their study, the approximately 300 cases analyzed involved critically ill patients. In contrast, dantrolene would appear to be very effective when used in patients with mild to moderate symptoms of stiffness and fever, especially when administered early in the presentation of NMS. For more severe cases, however, the trend is to favor a combination therapy of dantrolene with other medications, such as bromocriptine (Reulbach *et al.*, 2007; Pileggi and Cook, 2016).

1.11.3.2 Bromocriptine

Bromocriptine mesylate is a semisynthetic ergot alkaloid derivative with potent agonist activity in the postsynaptic dopaminergic receptors, indicated for the management of signs and symptoms of Parkinson. Bromocriptine has also been used, along with levodopa, as an adjuvant in progressive cases with motor complications, off-label to manage restless legs syndrome, and, in addition, may counteract the dopamine blockade attributed to antipsychotics in neuroleptic

malignant syndrome. It is uniquely available in an oral formulation (tablet or capsule) and can also be administered via feeding tube (Pileggi and Cook, 2016).

Although bromocriptine may worsen psychosis and contribute to changes in blood pressure, evidence of clinical effectiveness encourages the use of bromocriptine in NMS. Recrudescence of symptoms has been reported with rapid discontinuation of this medications, so typically bromocriptine discontinuation is done gradually. In addition to bromocriptine, other dopaminergic agents that are usually administered for the treatment of NMS are amantadine, apomorphine, and levodopa (used primarily in Parkinson's disease) (Mann *et al.*, 2003; Brian D Berman, 2011; Pileggi and Cook, 2016).

1.11.3.3 Benzodiazepine

Other pharmacologic agents that may have some relevance in the treatment of NMS are benzodiazepines, which may be useful in controlling agitation but may also improve symptoms and speed recovery in milder cases. Among benzodiazepines, scientific studies support as treatment for NMS the use of lorazepam, diazepam, and clonazepam (Brian D Berman, 2011; Pileggi and Cook, 2016).

Benzodiazepines can be administered by several routes: intra venous (IV), intramuscular (IM), intranasal, oral or buccal, etc. The route of administration and dose can be adapted depending on the clinical situation. The mechanism underlying their effectiveness is inversion of the GABAergic system that contributes to the occurrence of NMS symptoms. Moreover, benzodiazepines are effective in the management of some typical symptoms of NMS, including agitation control and in the reversal of catatonia. Furthermore, benzodiazepines, as well as amantadine, are effective in the treatment of neuroleptic-induced catatonia, which, in some cases, may precede the development of NMS(Mann *et al.*, 2003; Pileggi and Cook, 2016).

1.11.3.4 Amantadine

Amantadine is an antiviral drug, derived from adamantane, which also acts as an antiparkinsonian agent, even in combination with L-DOPA. In the management of neuroleptic malignant syndrome and symptoms, amantadine has been described as adjunctive treatment, especially in moderate or severe cases.

The mechanism of action underlying amantadine in the care of Parkinson's disease and drug-induced extrapyramidal adverse reactions is not well understood, although it would appear to cause an increase in dopamine release in the brain and also reduction in dopamine reuptake. It is also characterized by anticholinergic activity that would appear to drive D_2 receptors to high-affinity states, and also has NMDS (N-methyl-d-aspartic acid) receptor antagonist activity, which have been shown to ameliorate dyskinesias in association with levodopa. If NMS patients do not respond to the described medical therapies, an alternative that is used is electroconvulsive therapy, which would appear to be effective in improving some of the symptoms of NMS (Mann *et al.*, 2003; Pileggi and Cook, 2016).

1.11.3.5 Electroconvulsive Therapy (ECT)

Not in all reported cases of NMS, pharmacotherapy was efficacious. When drug therapies failed or did not yield the desired results, electroconvulsive therapy (ECT) was successfully used as an alternative approach. The use of ECT has been described to be highly successful in the treatment of catatonia and also in Parkinson's disease, probably due to increased dopamine synthesis and release. ECT is the preferred treatment for treated subjects whose hypermetabolic status is remedied but in whom psychosis or catatonia have not been successfully resolute (Mann *et al.*, 2003; Pileggi and Cook, 2016; Velamoor, 2017).

As in catatonia, it is certainly not surprising that ECT is also very effective and a life-saving therapy in the treatment of NMS during the acute phase, in drug-resistant cases and/or in the late course of the syndrome, and also in cases in which a persistent, enduring catatonic or parkinsonian state develops after NMS. Although NMS patients are not considered at risk of developing malignant hyperthermia during treatment with ECT, however, physicians should exercise caution; in fact, succinylcholine is known to cause arrhythmias and hyperkalemia, so other medications or treatments that cause muscle relaxation should be considered for such individuals. However, one of the greatest advantages of ECT is being able to treat not only NMS, but also the underlying psychosis that characterizes the patients (Mann *et al.*, 2003; Sienaert, van Harten and Rhebergen, 2019).

1.12 GENETIC PREDISPOSITION

As reported previously, although the frequency of NMS is rare, clinical case reports of syndrome occurrence within the same family have been reported, suggesting the presence of genetic-controlled constitutional factors, which may determine susceptibility to NMS development in some patients (Kawanishi, 2003; Ananth *et al.*, 2004; J. R. Strawn, Keck and Caroff, 2007).

Up to the present time, a restricted and selected number of genetic variants, primarily associated with variations in neurotransmitter receptor genes or metabolic activity, have been investigated in association with predisposition to NMS, albeit with inconclusive and conflicting findings (Kawanishi, 2003; Ananth *et al.*, 2004). In addition, most of the studies were conducted at the turn of the late 1990s and early 2000s, and almost all of them were on patients or controls belonging to the Japanese population, whereas for populations belonging to other ethnic groups only a few data are available mainly from case reports (Kawanishi, 2003; Del Tacca *et al.*, 2005; Živković *et al.*, 2010).

1.12.1 Drug targets

Although the mechanisms and etiopathogenesis underlying the onset of NMS are not yet fully elucidated, it is nevertheless widely believed that blockade of dopaminergic receptors at the level of the central nervous system, and in particular of the dopamine D_2 receptor, may be one of the main cornerstones for the development of the syndrome. Therefore, most studies regarding the possible genetic predisposition to NMS have focused on the dopamine D_2 receptor gene (*DRD2*) (Brian D Berman, 2011; Velamoor, 2017).

In 1995, Ram and colleagues studied the complete nucleotide sequence of the *DRD2* gene, encoding for the dopamine D_2 receptor, by sequencing it in its entirety. They examined 12 patients of different ethnicity who had developed NMS, including the two sisters previously described by Otani and colleagues a few years earlier as a case of familial NMS occurrence (Otani *et al.*, 1991; Ram *et al.*, 1995). Following the analysis conducted through the technique of denaturing gradient electrophoresis (DGGE), Ram and colleagues found, in a single NMS patient, the presence of a missense variant (rs1800496) which determines a nucleotide substitution (CCG→TCG) at codon 310 of exon 7 of the *DRD2* gene, also called Pro310Ser because it involves the aminoacid

substitution of proline to serine within the receptor protein sequence. However, because no control subject without the syndrome had been recruited for the study, and because the number of patients analyzed was small, the authors could not conclude whether the polymorphism they found could actually influence the onset of NMS (Ram *et al.*, 1995; Kawanishi, 2003).

A few years later, a group of researchers consisting of Suzuki *et al*. before, and Mihara *et al.* later, respectively investigated the possible association between the development of NMS and the presence of the TaqI A polymorphism, which appears to modifies the density and functionality of dopamine receptor D_2 (Suzuki *et al.*, 2001).

The TaqIA polymorphism (rs1800497, also known as Taq1a) is a missense variant that was previously mistakenly attributed to the *DRD2* gene, even though it is actually located over 10,000 bp downstream of the gene. In the recent years, thanks to the completion of the Human Genome Project (HGP), it was realized that this variant is located on the adjacent ankyrin repeat and protein kinase domain-containing protein 1 gene (*ANKK1*) and more precisely within exon 8, at position 11:113400106 (forward strand) (Neville, Johnstone and Walton, 2004; Green, Watson and Collins, 2015). However, the presence of this polymorphism is believed to affect the gene regulation of the D_2 receptor, lowering its receptor density, and is also associated with an early therapeutic response to certain categories of drugs, including antipsychotics, such as haloperidol (Jönsson *et al.*, 1999; Ananth *et al.*, 2004; Neville, Johnstone and Walton, 2004; Savitz, Colin A. Hodgkinson, *et al.*, 2013).

In the study, conducted by Suzuki and others, on a group of 15 Japanese patients with NMS presentation, the presence of the TaqIA polymorphism seemed to be associated with the development of the syndrome, because both the frequency of the TaqI A1 allele and the percentage of subjects carrying the A1 allele were considerably increased in individuals who had developed NMS (Suzuki *et al.*, 2001).

In the study published two years later by Mihara and colleagues, the association between the presence of three different polymorphisms attributed to dopamine receptor genes, and the occurrence of NMS, was investigate among 17 Japanese psychiatric patients with NMS (13 of whom had been previously studied by Suzuki *et al*.) in comparison with 163 schizophrenic patients who had never developed the syndrome. The three polymorphisms selected were TaqIA, the polymorphism -141C Ins/Del (rs1799732) in the promoter region of *DRD2*, the presence of which has been shown to cause lower density of dopamine D_2 receptor in the brain (Arinami *et al.*, 1997), and the Ser9Gly polymorphism (rs6280) located in the dopamine D_3 receptor gene (*DRD3*), which

may alter the receptor function. As in the previous study, the results confirmed that only the TaqI A1 allele appeared with a significantly higher frequency in NMS patients than in controls, whereas for the other two polymorphisms, no significant difference was found in the allelic and genotype frequencies between the two groups in comparison (Mihara *et al.*, 2003).

In the same years, two further studies by Kishida and colleagues were conducted analysing first one and then three different functional polymorphisms of the dopamine D_2 receptor gene, on a larger sample of Japanese patients (49 and 32 individuals with NMS, respectively) and their possible association with the development of neuroleptic malignant syndrome (Kishida *et al.*, 2003, 2004). The polymorphisms analyzed were only TaqIA first, and subsequently TaqIA together with -141C Ins/Del and Ser311Cys (rs1801028), a missense variant mapped to exon 7 of *DRD2* gene, which would appear to have an influence on the interactivity between D_2 receptor and its G protein. In neither study was any significant difference observed in the allele or genotype frequencies of the TaqI A1 allele between the two groups of subjects (affected and unaffected by NMS); similarly, no significant allelic or genotypic difference was found for the Ser311Cys polymorphism between the two groups. The -141C Del allele, although, was considerably more abundant in the NMS group than in the control group, suggesting that the -141C Ins/Del polymorphism, presumably in conjunction with other undetected elements, possibly predisposes toward susceptibility to the development of NMS (Kishida *et al.*, 2003, 2004).

The polymorphism -141C Ins/Del (rs1799732) has traditionally been so named because the gene on which it is localized was originally mapped in reverse strand. It is a variant consisting of an insertion/deletion of G (guanine), and it is located at chr11: 113475530 (forward strand) within the promoter region of *DRD2*, approximately 141 bases before the transcription start site (TSS) of the gene. A study conducted by Arinami's group, through a transactivation assay of a reporter gene under the control of the promoter region of *DRD2,* revealed a decrease in gene expression when the -141C deletion (G allele) was present (Arinami *et al.*, 1997). Moreover, according to Jönsson and colleagues, striatal dopamine D_2 receptor density has been demonstrated to vary significantly among healthy individuals, increasing considerably in carriers of -141C Del variant (Jönsson *et al.*, 1999). Furthermore, Pohjalainen and co-workers investigated, in healthy volunteer individuals, the Del allele influence on D_2 receptor binding properties in vivo by positron emission tomography (PET) and a D_2 receptor antagonist, finding no difference in D_2 receptor density among -141C Del allele carriers compared with noncarriers subjects. This finding suggests that genetic e -141C Ins/Del polymorphism would not influence the D_2 receptor expression level in vivo, in contrast to the results published by Arinami and Jönsson (Pohjalainen *et al.*, 1999).

As already anticipated, the relationship between the presence of this polymorphism and susceptibility to the development of NMS has been analyzed in two different studies, published consecutively, both conducted on the Japanese population, which reported opposite results. In fact, while in the first study there were no differences in the allelic and genotypic frequencies of the variant examined among controls and NMS patients, the second one a significantly high frequency of the -141C deletion (G allele) was reported in the cohort of NMS patients compared with the controls group (Mihara *et al.*, 2003; Kishida *et al.*, 2004).

Other drug targets that have been genetically investigated in relation to neuroleptic malignant syndrome are serotonin receptors. Serotonin syndrome has been reported to have common physiopathological features with NMS, and sometimes the two syndromes can occur with indistinguishable presentations. Therefore, it was suggested that the serotonergic system might be involved in the etiopathogenesis or the onset of NMS (Kawanishi, 2003; Rusyniak and Sprague, 2005; J. R. Strawn, Keck and Caroff, 2007; Steele, Keltner and McGuiness, 2011). Kawanishi and co-workers investigated the frequency of polymorphisms in genes encoding for various serotonin receptor subtypes, and their possible interaction in predisposing toward susceptibility to NMS development. Specifically, they determined the frequencies of three missense variants, among 29 NMS patients compared to a control group, consisting of 94 antipsychotic-treated schizophrenic subjects who had no experience of NMS and 94 healthy individuals, all of Japanese ethnicity: one polymorphism, known as Arg219Leu (rs200894913), belonging to the gene encoding for the 5-hydroxytryptamine receptor 1A (*HTR1A*), and the other two, located on the 5-hydroxytryptamine receptor 2A (*HTR2A*) and known as Thr25Asn (rs1805055) and His452Tyr (rs6314). However, no allelic variant was found among NMS patients. However, no differences in allelic or genotypic frequency were found among NMS patients and controls (Kawanishi *et al.*, 1998; Kawanishi, 2003).

The clinical manifestations of NMS can also be easily confused with those of malignant hyperthermia (MH) thus, some authors have proposed that there may be an overlap between the etiopathogenetic mechanisms underlying the two syndromes. In this regard, some variants belonging to the *RYR1* gene, historically studied and analyzed in association with MH, were investigated by Miyatake and colleagues in 10 patients of Japanese origin who had developed NMS. Of the six variants analyzed and previously associated with susceptibility to the development of MH, no mutations were found between NMS patients and controls (Miyatake *et al.*, 1996). In another study conducted more recently by Sato and other researchers, some possible genetic variants of the *RYR1* gene were investigated through the post-mortem diagnosis of 11

psychiatric patients, suspected to have died of NMS following autopsy examinations. As a result of genetic analysis, two mutations located on the *RYR1* gene were identified, one (R4645Q) already previously reported in MH patients, while the other mutation (A612T) was new. Although the presence of *RYR1* gene mutations and their association with psychiatric patients suspected to have died of NMS remains unclear, these findings provide the first successful identification of *RYR1* mutations in association with susceptibility to NMS, even if suspected (Sato *et al.*, 2010).

1.12.2 Metabolic target

From a pharmacogenetic perspective, cytochrome P450 (CYP) 2D6 is the most studied major hepatic oxidative enzyme for drug metabolization. It is involved in the metabolism of a large number of medications, including antipsychotics drugs (such as haloperidol, risperidone, zuclopenthixol) some antiemetics and most antidepressants. This enzyme is encoded by the gene (*CYP2D6*) which is known to be highly polymorphic, having more than 80 allelic variants described so far (for updates https://www.pharmvar.org/genes). As such, the enzymatic activity of the resulting isophorms correlates with several phenotypes: extensive (EM), poor (PM), intermediate (IM), and ultrarapid (UM) metabolizers (Bertilsson *et al.*, 2002; Kawanishi, 2003). It is well known that *CYP2D6* enzymatic activity differs among various ethnic populations, being also strictly dependent on the distribution and frequency of *CYP2D6* gene polymorphisms. In recent decades, especially between the 1990s and 2000s, several studies have focused on the genotyping of *CYP2D6* polymorphisms, suggesting a correlation between the presence of genetic variants and defective CYP2D6 isoenzyme activity (Iwahashi, 1994; Kato *et al.*, 2007).

Kawanishi and colleagues, analyzing the genotype of two Japanese patients who had previously developed NMS, detected in both individuals the presence of the *CYP2D6*10* allele in homozygosity, which encodes a form of reduced activity of the enzyme and consequently confers a poor- metabolizer phenotype, and hypothesized that the onset of the syndrome could be related to this genotype (Kawanishi, 2003). Shortly thereafter, the same research group compared the frequency of *CYP2D6*10* variant among NMS patients and control subjects, with the aim of examining the possible association between unstable CYP2D6 enzymatic activity and NMS, but with negative results (Kawanishi, 2003).

Kato and colleagues, in two consecutive studies, investigated the potential relationship between the presence of *CYP2D6* polymorphisms and the occurrence of NMS (Kato *et al.*, 2005, 2007). In the first preliminary report, two schizophrenic patients both treated with antipsychotic

medications, who had previously experienced NMS, were tested for a *CYP2D6* gene deletion allele (*CYP2D6*5*), the presence of which is correlated with a decrease in enzyme activity and may lead to accumulation of drugs. As a result of a restriction fragment length polymorphism (RFLP) analysis on an amplicon of the *CYP2D6* gene, it was detected the presence of the genotype *5/*10 in the first patient, while the other had a genotype *1/*5 (Kato *et al.*, 2005). In the following study, intended to examine the effects of drug metabolism on the incidence of NMS, the research team expanded the cohort of patients who had experienced NMS to 53, and also included a control group of 112 healthy subjects, on whom to perform the genetic analysis, all of Japanese descent. The results confirmed the findings of the previous study, explicitly that the prevalence of *5 alleles in the NMS patients was higher than that in the control group, however no statistically significant difference was reached. Moreover, a group consisting of 29 patients out of 54 total appeared to have developed the syndrome following therapy with drugs that are targets of the CYP2D6 enzyme, and among these the frequency of the *CYP2D6*5* alleles was significantly higher than in controls. Additionally, in only one patient was found the *CYP2D6**4 allele, and in another one a duplicate allele, whereas no association was identified among the incidence of the *CYP2D6**10 alleles and the occurrence of NMS. These results, however, seem to suggest that the CYP2D6*5 allele may influence vulnerability to the development of NMS (Kato *et al.*, 2007).

Another study, conducted on both drug target and metabolic target, was performed by Živković and coworkers. Two different subjects came to the attention of the research team, both with clinical presentations of three consecutive NMS episodes. The first patient developed NMS during a therapy with combination of medications, consecutively: haloperidol, promazine and fluphenazine the first time, fluphenazine and perazine the second time and clozapine, promazine and valproic acid the last time. The second patient also developed NMS while taking the following therapy: haloperidol and lithium carbonate the first time, then only risperidone, and finally clozapine for the second and the third time, respectively. Pharmacogenetic analysis was performed on TaqIA *DRD2* and *CYP2D6* polymorphisms, for both individuals. Taql A polymorphism analysis revealed the presence in heterozygosity of the A1 allele (genotype A1A2) only in the first patient. Moreover, *CYP2D6* genotyping of *1*3*4*5*6 alleles in both patients revealed no evidence of poor metabolizer phenotype. Again, however, the presence in heterozygosity for CYP2D6*4 (genotype *1/*4), was detected only in the first of the two patients. Therefore, polymorphisms at metabolic genes, such as *CYP2D6*, could have clinical significance because they could lead to toxicity and the occurrence of adverse side effects even following standard dosage administration of antipsychotics (Živković *et al.*, 2010).

All the studies conducted so far, concerning pharmacogenetics or the analysis of variants and gene polymorphisms in relation to neuroleptic malignant syndrome, have given partial results, also because of the limited number of patients NMS, and have often provided conflicting results between them. In addition, most of the studies on gene frequencies, have been conducted only on the Japanese population. Therefore, further analysis and extensive studies are required to identify genetic biomarkers and establish a possible correlation between genetic variants, polymorphisms and mutations and the susceptibility to the development of NMS, as well as it is essential to implement genetic analysis on other populations, in addition to the Japanese one (Kawanishi, 2003; Sato *et al.*, 2010; Živković *et al.*, 2010).

1.13 WHOLE GENOME SEQUENCING AND OMIC APPROACH

About twenty years ago, in February of 2001, was firstly release the draft of the nuclear human genomic sequence.

Accordingly the latest release of the human genome GRCh38.p13 (Genome Reference Consortium Human Build 38) INSDC Assembly GCA_000001405.28, Dec 2013, the entire sequence of the human genome consists of 3,096,649,726 base pairs, 20,471 coding genes and 24,842 non-coding genes (4,865 small non-coding genes and 17,756 long non-coding genes, 2,221 misc non-coding genes) and 246,685 gene transcripts (http://www.ensembl.org/Homo_sapiens/Info/Annotation).

In human somatic cells there are 23 pairs of homologous chromosomes, for a total of 46: more precisely, there are 22 matched pairs of autosomal and the two sexual chromosomes, X and Y determining the genetic sex of male (XY) or female (XX). Only a small fraction of all DNA, consisting of about 1.5% of the 3.1 billion bp of the human genome, is made by coding genes that are transcribed and processed into exons, whereas another fraction, the introns, are spliced out. The set of coding, non-coding and regulatory sequences constitutes the "functional" genomic DNA, which corresponds to about 25% of the entire genome. The remaining 75% of the genome, on the other hand, is defined as extragenic and, of this, more than 50% consists of repetitive DNA (Moy, 2007; Wheeler *et al.*, 2008; Butler, 2010; Salzberg, 2018).

It has been estimated that more than 99.7% of the human genome has the same sequence in all individuals, thus each individual is different from another one for only the 0.3% of the genome (about 10 million nucleotides). It is in this fraction that lies the genetic variability that makes each subject unique, and distinguishes it from others, with the exception of monozygotic twins, which are indistinguishable from the genetic point of view.

The diversity between individuals, due to the genetic variability, is a fundamental biological element upon which evolution is based and which outlines the heritable foundation of phenotype and can be attributable to changes in DNA sequence that are transmitted to descendants only if they occur in the germ line. The genetic variants have been classified in genomic structural variants (bigger than 50 base pairs) or small variants. Single Nucleotide Variants (SNVs) also named Single Nucleotide Polymorphisms (SNPs), small insertions or deletions (INDELs), Variation Number of Tandem Repeats (VNTRs) are, generally considered small variants smaller than 50 base pairs,

whereas segments larger than 50 base pairs that are either deleted, flipped, translocated or amplified, are known as Copy Number Variants (CNVs) and Structural Variations (SVs) (Barnes, 2010; Butler, 2010; Ng and Kirkness, 2010). All these genomic variants may occur in the germline, that are inherited to the progeny, somatic that may occur in somatic cells, or inborn.

Data about these genetic variations can provide precious and more complete information into the functional range of a gene, the pathways in which it is involved, and the functions of a protein or regulatory elements. Moreover, several studies investigating genetic variation in association with disease development and interindividual variation in drug response, have provided great basis for the impact of "genomic medicine" (Barnes, 2010).

The term “omics” or “omic sciences” (i.e.: genomics, transcriptomics, proteomics, metabolomics) refers to all those disciplines that have as their object of study the characterization and quantification of biological molecules, in order to outline the structure, functions and functional dynamics that characterize an organism. Anything “omics” is also often referred to by the specification “wide” or “whole”, such as genome-wide association studies, whole exome sequencing or genome sequencing, which are just examples that implicitly refer to omic sciences.

Genetics and genomics are not the same thing: genetics is about the single gene, while genomics is about all the genome of the organism, compares the genetic heritage of organisms belonging to different species (comparative genomics), describes the functions and interactions of genes and their products (functional genomics) and turns its gaze even to those DNA sequences that do not contain genes and that seem to be "junk" and whose meaning is still being questioned. All that is omics, in fact, is global, inclusive, and provides a massive amount of information (Collotta and Ventriglia, 2018).

Pharmacogenomics (PGx) is an emerging approach that involves several fields of "precision" medicine, with the aim of tailoring drug dosage and selection to the genetic characteristics of the patient, minimizing adverse drug reactions, and continuing to identify new medications and therapeutic treatments customize for disease (Ampong, 2019; Cecchin and Stocco, 2020).

In recent years, many international collaborative initiatives are underway in order to implement research and knowledge regarding pharmacogenomics. However, the pharmacogenomic markers discovered and validated so far can explain only a small fraction of the clinical differences and variation in pharmacological treatment outcome observed among patients, and the routine use of pharmacogenomics in clinical practice remains limited. Therefore, new study strategies are needed, including the study of the pharmacogenomic features of rare genetic variants that were

previously overlooked, but may instead be of great interest in explaining a large portion of the interindividual variability in drug metabolism.

Moreover, the development and implementation of pharmacogenomics in clinical practice should focus not only on the study of rare genetic variants, but also on the search for new pharmacogenomic markers to improve the safety and efficacy of drugs, and this could have a positive impact on the lives of patients who are resistant to drug treatments, or psychiatric individuals who do not respond to treatment with antipsychotic drugs, or individuals who have had a history of serious adverse effects as a result of taking antipsychotic medications (Ampong, 2019; Cecchin and Stocco, 2020).

Whole genome sequencing and next-generation sequencing technologies, providing data on the totality of variants, from the rarest to the least rare, of each individual whose genetic makeup is sequenced, as well as the most complete information on structural variations, are moving knowledge into a new phase focused on the individual genome and full disclosure of individual variation and, potentially, are the best solution for human genetic association studies that aim to identify the genetic basis of certain syndromes and diseases, as well as susceptibility to adverse drug reactions (Barnes, 2010; Ng and Kirkness, 2010).

2 AIM OF THE STUDY

Neuroleptic Malignant Syndrome (NMS) is a rare but severe and potentially fatal idiosyncratic reaction that can occur in response to administration of central dopamine (D_2) receptor antagonists (such as antipsychotic drugs) or the rapid withdrawal of a dopamine agonist. The syndrome is characterized by hyperthermia, autonomic instability, muscle stiffness, altered consciousness, variable blood pressure, creatine kinase elevation. Environmental parameters, polypharmacy therapy, body stress and other conditions have been considered risk factors that can concur in triggering the insurgence of this syndrome. NMS has been commonly associated with neuroleptic medications, including first-generation or "typical" antipsychotics, and second-generation or "atypical" antipsychotics, however assumption of antidepressants, lithium, illicit substances of abuse (such as amphetamine or cocaine) or the sudden withdrawal of dopaminergic medications have also been reported. NMS is a diagnosis of exclusion, and according to IEC (International Expert Consensus) NMS diagnostic criteria and the 5th edition of the *Diagnostic and Statistical Manual of Mental Disorders*, key points for a correct NMS diagnosis are the presence of causative agents and the absence of other etiologies.

The pathophysiology underlying NMS remains relatively unknown, however evidence support the hypothesis that a reduction in central dopaminergic activity due to D_2 receptor blockade within the nigrostriatal, hypothalamic, mesolimbic, and mesocortical pathways may explain some of the typical clinical features of the syndrome. Serotonergic and cholinergic systems have also been considered contributors to NMS, either independently or in conjunction with the dopaminergic system.

No genetic biomarkers for NMS predisposition have been clearly identified so far, nevertheless candidate genes have been proposed, with conflicting results, in association with NMS susceptibility, including dopamine, serotonin, and cytochrome P450 2D6 enzymes (Iwahashi, 1994; Kawanishi *et al.*, 1998; Kawanishi, 2003; Kishida *et al.*, 2004; Kato *et al.*, 2007).

The first aim of this project was to investigate three genetic polymorphisms of *DRD2* (dopamine receptors) that were previously reported with unconclusive results to be associated with NMS predisposition. Differently from the studies on a Japanese cohort, this study was conducted in collaboration with Dr. Valeria Margherita Petrolini, Centro Nazionale Antiveleni, IRCCS Maugeri, Pavia, on a cohort of well-characterized south European non-Finnish patients who developed NMS upon antipsychotic therapy.

The second aim of this project was to perform a whole genome sequencing of NMS patients to discover genomic traits associated with the NMS susceptibility.

3 MATERIALS AND METHODS

3.1 AFFILIATION OF THE STUDY

A case-control study of a prospective cohort was conducted with the purpose of identifying possible genetic variants of the *DRD2* gene associated with neuroleptic malignant syndrome (NMS).

This study was a collaborative effort between Drs. Ornella Pastoris, Laboratory of Pharmacogenetics and Experimental Toxicology, University of Pavia and Fiorenzo A. Peverali, Institute of Molecular Genetics "Luigi Luca Cavalli-Sforza", National Council of Research, Pavia (http://www.igm.cnr.it/).

The recruitment of patients and healthy individuals and diagnosis of NMS were supervised by Drs. Valeria M. Petrolini and Carlo Locatelli, Poison Control Centre (PCC) and National Toxicology Information Centre (CNIT) of the IRCCS ICS Maugeri Hospital in Pavia (https://www.icsmaugeri.it/per-i-ricercatori/laboratori-ricerca/servizio-di-tossicologia-centro-antiveleni-e-centro-nazionale).

Statistical analysis of genetics data was supervised by Drs. Maria Cristina Monti and Simona Villani, Clinic Epidemiology and Biostatistics Laboratory, University of Pavia (https://spmsf.unipv.it/dipartimento/unita/biostatistica-ed-epidemiologia-clinica/presentazione.html).

3.2 RECRUITMENT OF PATIENTS

Data of patients was recorded by the PCC – CNIT unit, IRCCS Maugeri, Pavia, Italy, on the basis of diagnosis or presumed diagnosis at hospital admission or during hospital stay and anonymized in compliance with the current privacy regulations (GDPR).

The study was approved by the Ethics Committee of *ICS Maugeri SpA SB-IRCCS, Pavia (Italy)* (https://www.icsmaugeri.it/il-comitato-etico).

Initially, a cohort of 39 patients who experienced hyperthermia triggered by antipsychotic drugs or illicit substances by the PCC – CNIT unit was recruited. Of these, a total of 17 patients with NMS diagnosis triggered by antipsychotic drugs was investigated in the present study. A cohort of 70 healthy individuals were also recruited in the present study.

Peripheral blood samples of patients and healthy individuals were collected upon informed consent (eventually obtained from the physician who oversaw the patient, if he/her was unconscious), transported to the PCC – CNIT unit at controlled temperature, anonymized and stored or immediately processed for the genetic and genomic analysis (see below).

3.3 INCLUSION CRITERIA OF THE STUDY

The diagnosis of NMS was conducted based on the occurrence of most of the following symptoms and signs, according to the guidelines of the International Expert Consensus for NMS diagnosis (Gurrera *et al.*, 2011, 2017):

1. recent exposure to a dopamine antagonist (or dopamine agonist withdrawal);
2. hyperthermia (>38.0 °C for more than 6 hours, resistant to pharmacological treatment with antipyretics and NSAIDs);
3. muscle rigidity;
4. rhabdomyolysis with creatine kinase elevation (at least 3 times upper the normal limit [171 U/l for males, 145 U/l for females]);
5. signs of SNS lability (BP elevation, ≥ 25% baseline; BP fluctuation, ≥ 20 mmHg (diastolic) or ≥ 25 mmHg (systolic) change within 24 h);
6. signs of hypermetabolism (tachycardia [≥ 25% above baseline] and tachypnoea [≥ 50% above baseline];
7. negative workup for other aetiologies.

Beside the above listed of symptoms and clinical signs, the trigger, i.e. antipsychotics vs illicit substances, was considered a critical issue for differential diagnosis between NMS vs NMS-like, respectively.

The 70 healthy individuals (20 males and 49 females; mean age 42 years, SD 15.5) without known history of drug-induced hyperthermia or NMS, were enrolled after entering the hospital for a routine blood examination.

All the individuals of the study were of European non-Finnish ethnicity.

3.4 GENETIC ANALYSIS

3.4.1 Sample collection and genomic DNA preparation

About 3-10 ml samples of peripheral venous samples were collected into Vacutainer® tubes containing EDTA and stored at -20°C until analysis.

Genomic DNA of each patient and healthy individual was purified from whole blood using the QIAamp Blood Midi Kit (Spin Protocol) (QIAGEN, Hilden, Germany – Europe). This protocol allows the purification of genomic DNA from up to 2 ml of whole blood, thereby permitting the extraction of the highest concentration or maximum yield of DNA samples, desirable for some applications such as whole Exome or whole Genome sequencing.

All the procedures of DNA purification were performed according to the protocol provided by the manufacturer (**Figure 3.1**):

1. QIAGEN Protease K solution (100 µl) was pipetted to the bottom of a 15 ml centrifuge tube.
2. Whole blood sample (from 300 µl to 1 ml) of was transferred from the EDTA-Vacutainer tube to 15 ml polypropylene centrifuge tube and mixed briefly.
3. 1.2 ml of Buffer AL was added to the sample and then the sample was mix thoroughly by inverting the tube 15 times, followed by additional vigorous shaking for at least 1 min.
4. The sample was then lysed by incubation at 70 ºC for 10 minutes.
5. After the incubation time, 1 ml of ethanol (96-100 %) was added to the sample, and then the tube was mix thoroughly by inverting it 10 times, followed by additional vigorous shaking.
6. The solution was carefully transferred into the QIAamp Midi column, avoiding moistening the edge and centrifuged at 1850 x g (3000 rpm) for 3 minutes.
7. The QIAamp Midi column was sequentially washed by adding Buffer AW1 (2 ml) and Buffer AW2 (2 ml) and centrifuged at 4500 x g (5000 rpm) for 1 minute and for 15 minutes after each washing step, respectively.
8. The QIAamp Midi column was placed onto a clean 15 ml centrifuge tube.

9. Buffer AE (200 µl) equilibrated to room temperature (15–25°C) was added directly onto the membrane of the QIAamp Midi column and incubated at room temperature for 5 minutes before eluting the genomic DNA by centrifugation at 4500 x g (5000 rpm) for 2 minutes.
10. The elution step with Buffer AE (200 µl) was repeated to further purify residual gDNA trapped on the QIAamp Midi column.

Genomic DNA (gDNA) was quantified by spectrophotometric analysis at 260 nm with a NanoDrop® ND 1000 instrument (NanoDrop, Thermo Fisher, MD, USA).

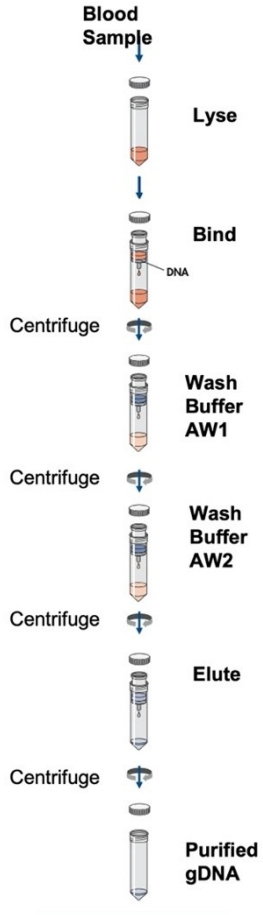

Figure 3.1: DNA Purification procedure. A schematic representation of the gDNA purification procedure from whole blood, according to the protocol provided by the manufacturer of the DNA extraction kit.

3.4.2 Amplification of genomic target sequence by PCR

To amplify genomic regions of the *DRD2* and *ANKK1* loci carrying the SNPs of interest, PCR (Polymerase Chain Reaction) was employed on the human genomic DNA purified from whole blood samples of patients and healthy individual.

A typical PCR reaction was as following:

gDNA template about	0.2 to 0.25 µg	
Polymerase Master Mix	7 µl	
GOTAQ Hot Start Green Master Mix G2 (Fisher Molecular Biology, Trevose, PA - USA)		
primer forward (final conc):	0.25 µM	1.5 µl from 2.5mM stock solution
primer reverse (final conc):	0.25 µM	1.5 µl from 2.5mM stock solution
water to a final volume of	35µl	

The PCR program is detailed in **Table 3.1** for each primer set:

- Initial step of denaturation at 95-98°C for 2min
- 40 cycles of:
  - Denaturation step at 95-98°C for 1 min
  - Annealing step at 56-74°C for 30 sec
  - Extension step 72-74°C for 30 sec
- final step of extension at 72°C for 5-10 min

Site	Primer Name	Oligonucleotide sequence (5'-3')	T_m	Reaction Mix composition	PCR conditions	PCR product lenght
Primer set 1 (rs1800497)	NM1F	CCTTCCTGAGTGTCATCAAC	57.3 °C	35 µl reaction mix containing 2-250 ng of genomic DNA, 0.5 X GOTAQ Hot Start Green Master Mix G2, 0.25 µM of each primer, and nuclease-free water up to volume.	Initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 40 sec, annealing at 56°C for 30 sec, extesion at 72°C for 1 min and final extension at 72°C for 5 min.	~236 *bp*
	NM1R	ACGGCTCCTTGCCCTCTAG	61.0 °C			
Primer set 2 (rs1799978 rs1799732)	677Fw	ACTGGCGAGCAGACGGTGAGGACCC	71.2°C	35 µl reaction mix containing 2-250 ng of genomic DNA, 0.5 X GOTAQ Hot Start Green Master Mix G2 and 0.25 µM of each primer, and nuclease-free water up to volume.	Initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 98°C for 20 sec, annealing and extesion at 74°C for 2.5 min and final extension at 72°C for 10 min.	~303 *bp*
	676Rv	TGCGCGCGTGAGGCTGCCGGTTCGG	74.5°C			

Table 3.1: Primers and PCR Reaction conditions. Primer sets, PCR settings and expected size in bp of the amplicons targeting the DRD2 locus.

The PCR reactions were performed in the thermal cycler (iCycler™, Bio-Rad, Hercules, CA - USA). PCR products were purified from the residual primers by Gel/PCR Extraction & PCR Clean Up kit (Fisher Molecular Biology, Trevose, PA - USA) (see below).

3.4.3 PCR purification

PCR products were purified using the Gel/PCR Extraction & PCR Clean Up kit (Fisher Molecular Biology, Trevose, PA - USA) according to the manufacturer instructions (**Figure 3.2**).

1. Up to 100 µl of PCR product (20 µl) were transfer into a microcentrifuge tube and 5 volumes of FSDF Buffer were added. Then, the reaction was mixed well by vortexing.
2. A FSDF column was placed into a collection tube.
3. The sample mixture was transferred carefully to the FSDF column and centrifuged at 11,000 x g for 30/60 seconds, then the flow-through was discarded.
4. Wash Buffer (750 µl) was added to the FSDF Column and centrifuged at 11,000 x g for 30/60 seconds, then the flow-through was discarded.
5. The sample mixture was centrifuged again at full speed (18,000 x g) for an additional 3 minutes to dry the column matrix.
6. The FSDF column was placed to a new microcentrifuge tube.
7. Elution Buffer (40 µl) was carefully added on the membrane of the FSDF column and incubated for at least 1 minute at room temperature before spinning at 18,000 x g for 1 minute in Microfuge to elute the DNA.

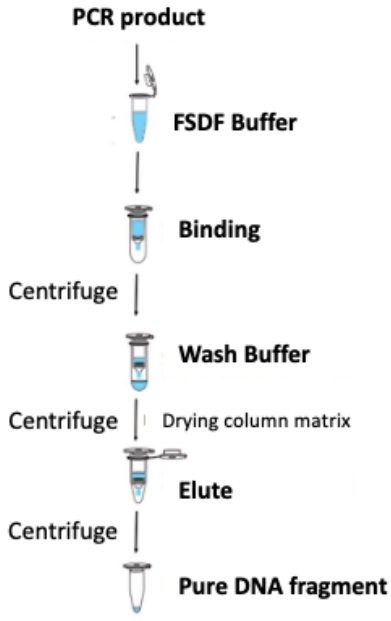

Figure 3.2: PCR product purification procedure. The picture shows a schematic representation of the PCR product purification procedure according to the protocol provided by the manufacturer.

3.4.4 SNP Genotyping by RFLP and Sanger sequencing

To identify the variant alleles of the *ANKK1* and *DRD2* genes, PCR products (about 1/5) were digested with the Taq$^{\alpha}$1 (for rs1800497), MaeIII and BstNI (for rs1799978 and rs1799732, respectively) (NEB, New England Biolabs) restriction enzymes (RE) according to manufacturer instructions, respectively.

About 1/5 of the RE was fractionated onto ethidium bromide pre-stained 3% TBE-agarose gel electrophoresis at 80 volts for 60 minutes. The size of the amplicons and the relative digested products was estimated by comparison with a standard molecular size marker, 100-bp or 50-bp DNA ladder (Fisher Molecular Biology, Trevose, PA - USA).

Electrophoresis image of the agarose gel was collected by the GelDoc imaging system (Bio-Rad Laboratories Inc, CA, USA).

PCR products were also Sanger sequenced by Mix2Seq kit Eurofins Genomics (https://www.eurofinsgenomics.eu/). About 15 µl of purified DNA at 1 ng/µl concentration were added to the Mix2Seq tubes with 2 µl of a specific Primer (forward or reverse) at the final concentration of 10 pmol/ µl (10 µM). Sequencing data were provided by web in file formats .ab1, .scf, .seq, .phd.1, .pdf and FASTA.

The sequences were aligned to human reference genome GRCh38.p13 by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.5 WHOLE GENOME SEQUENCING

3.5.1 Samples preparation and shipment of gDNA

Whole genome sequencing was carried out by Macrogen (www.macrogen.com).

Genomic DNA (in TE buffer or DEPC water) was aliquoted in a 1.5~2.0ml microcentrifuge tube at a minimum concentration of 50ng/μℓ.

The sample order form was filled with the requested information and the sealed microfuge tubes were put into Falcon 50-mL disposable screw-capped tube filled with clean tissue paper to prevent hard shaking during the shipment of gDNA at room temperature (**Figure 3.3**).

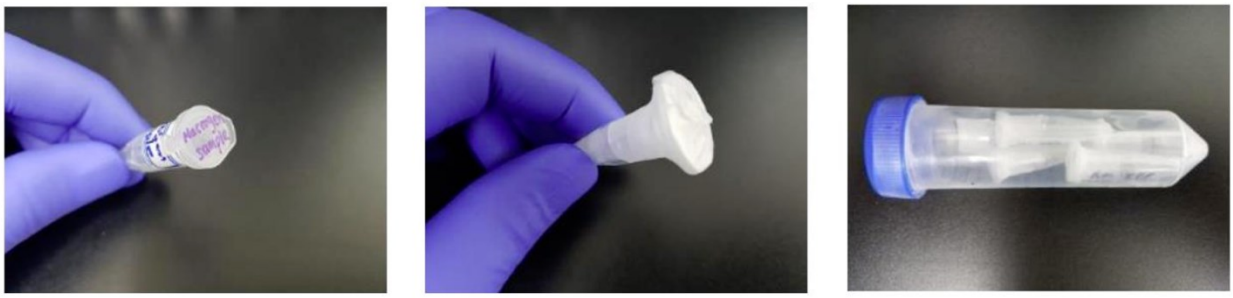

Figure 3.3: DNA preparation for shipment. The figure shows the guidelines for sample preparation and package for shipment to the sequencing company (www.macrogen.com).

3.5.2 Whole Genome Sequencing general information

The gDNA samples were prepared according to the Illumina TruSeq Nano DNA library preparation guide and sequenced by "Illumina HiSeq X sequencer".

Library preparation was as follows (**Figure 3.4**):

1. DNA fragmentation: genomic DNA was fragmented by covaris systems, which generates double-strand DNA (dsDNA) fragments with 3' or 5' overhangs, to obtain a final library with an average insert sizes of 300-400 bp.
2. End repair and size selection: the fragmented double-stranded DNA fragments were blunt ended by the enzyme mix containing the 3' to 5' exonuclease to remove the 3' protrusions, and the polymerase to fill in the 5' protrusions. Next, size selection of library fragment was carried out by using different bead ratios.

3. Adenylation of 3’ end: the nucleotide adenine was added to the 3' ends of the blunt fragments to prevent the coupling among fragments during the adapter binding reaction and prepare the TA ligation step of the adapter to the fragment.

4. Adapters ligation: multiple indexing adapters were ligated to the ends of the DNA fragments to prepare them for hybridization on a flow cell.

5. Library validation: quality control analysis on the sample library and quantification of the DNA library models were then performed by the company.

Illumina sequencing is based on a single "bridging" amplification reaction that takes place on the surface of the flow cell, which contains millions of unique clusters and is loaded onto the sequencer for automated extension and imaging cycle, through sequencing-by-synthesis.

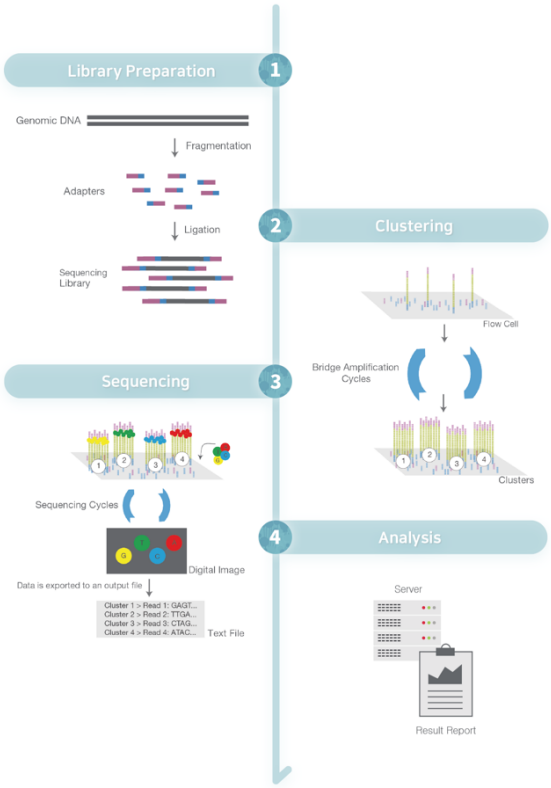

Figure 3.4: Schematic representation of library preparation. The figure schematically summarizes the basic steps involved in sample and library preparation for sequencing.

Whole Genome Sequencing was carried out at 30X coverage. WGS of each patients generate the sequence of about 3.2 billion base pairs, about 21 thousand genes, about 5 million SNPs and INDELs, almost 10 thousand structural variants (SVs) and about 900/1000 copy number variants (CNVs).

In this study work, an in-depth analysis of variants in copy number (CNVs), gains and losses, distributed in both coding and non-coding regions and structured variants (SVs) were performed, deferring the analysis of SNPs and INDELs shorter than 50bp to a more long-term study.

3.6 BIOINFORMATIC ANALYSIS

Sequencing data are provided in different file formats (fastq.gz, .bam, .bam.bai, .vcf, .xlsx, .vcf.gz, .vcf.gz.tbi) which must then be processed through bioinformatics analysis.

The first step was to check quality control report and the raw sequence data from the high-throughput sequencing pipelines using FastQC files, to make sure that the raw data were of good quality and not affected by any sequencing bias that could invalidate the analysis.

The extrapolation of the data concerning the Copy number variations examined in this thesis, the comparison and extraction of the elements shared among all the patients and among the various subcategories of patients, was performed through the command line interface (unix "Terminal").

To manipulate files .vcf (Variant Calling Format) of the CNVs, the software "BCFtools" version 1.15 was installed into the Unix Terminal.

BCFtools is a set of utilities that allows you to execute variant calls, in the VCF formats and its binary counterpart BCF. All commands can be executed seamlessly with both VCFs and BCFs, both uncompressed and compressed with BGZF. BCFtools manual can be provided at https://samtools.github.io/bcftools/bcftools.html.

Firstly, the .vcf files of the CNVs and the SVs of each patient was compressed and indexed; this operation in general has to be done every time several VCFs are read simultaneously. Subsequently, the CNVs files of the 11 patients were concatenated together (put together in one file) in .txt format; the new file was then sorted (i.e., all lines in the one file containing all patient information were organized); unique and shared rows were filtered and counted; finally, only information that were shared among the 11 patients were extracted. The same command pipeline was also used to extract the CNVs shared among the 2 subgroups of the 7 SGA patients and the 6 FGA patients, and among the 2 subgroups of the 7 G-carrier patients and 4 G-non carrier patients. At the end of this operation, a *.tsv* file was created to be easily read by Excel (**Figure 3.5**). As for the SV files, after being compressed and indexed, they were filtered through the BCFtools Isec command, which create intersections, unions and complements of VCF files (**Figure 3.6**).

```
conda activate bcftools
for f in *.vcf; do bgzip $f;done
for f in *.vcf.gz; do tabix -p vcf $f;done
cat *.txt| sort | uniq -c | grep 11 -w -n >sharedCNV_all.tsv
```

Figure 3.5: BCFtools command for CNVs. In figure the strings of command used in the software bcftools for the bioinformatic analysis of CNVs are detailed.

```
conda activate bcftools
for f in *.vcf; do bgzip $f;done
for f in *.vcf.gz; do tabix -p vcf $f;done
bcftools isec -n~11 -c all samples.vcf.gz > Shared_SV.vcf
```

Figure 3.6: BCFtools command for SVs. In figure the strings of command used in the software bcftools for the bioinformatic analysis of SVs are detailed.

The shared CNVs and SVs were displayed on the Human Genome Reference DNA, GRCh38.p13 by Ensembl database (https://www.ensembl.org/).

Shared CNVs and SVs mapped on reference Genome whose genomic frequency was archived in the 1000 Genome Project phase 3 and/or described in the DGVa (Database of Genomic Variants archive, https://www.ebi.ac.uk/dgva/) were considered typical genetic traits of the South European non-Finnish population and excluded from further investigation.

"DECIPHER" (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) (https://www.deciphergenomics.org) was also consulted to determine whether the identified copy number variants, gains and losses, were already associated to diseases or pathological symptoms and signs.

The genetic information of the SNVs, INDELS and CNVs reported in this study were obtained from the human GRCh38.p13 genome assembly by genome browser Ensembl Release 105 (http://www.ensembl.org/index.html), the NCBI Gene (https://www.ncbi.nlm.nih.gov/gene) and OMIM (https://www.omim.org/).

Data regarding genetic variants, structural variants, copy number variations, polymorphisms and their frequencies were extracted from several databases such as dbsSNP (https://www.ncbi.nlm.nih.gov/snp/), gnomAD v2.1.1 (https://gnomAD.broadinstitute.org/) and the Pharmacogene Variation (PharmVar) Consortium (https://www.pharmvar.org/gene/CYP2C9).

General information on drugs, small molecule and substances involved in the study (mainly taken by NMS patients) were obtained through consultations of specific databases, such as DrugBank (https://go.drugbank.com/drugs) and PubChem (https://pubchem.ncbi.nlm.nih.gov).

Information regarding the interactions of antipsychotic drugs with their targets, and in particular the genes coding for the involved receptors, metabolism enzymes, and transporters, were obtained DrugBank, Stitch Database Version 5.0 (http://stitch.embl.de) and IntAct Portal (https://www.ebi.ac.uk/intact/home).

Information about proteins and their expression data were from Uniprot (https://www.uniprot.org) and The Human Protein Atlas (https://www.proteinatlas.org), a source of data also on RNA expression and from which the list of genes expressed in the central nervous system were extrapolated. (Uhlén *et al.*, 2015).

IntAct and String (https://string-db.org) were consulted for protein-protein interactions, while information about the pathways involved in signal transduction were from Reactome Pathway Database (https://reactome.org) and KEGG (Kyoto Encyclopedia of Genes and Genome) Pathways (https://www.genome.jp/kegg/).

4 RESULTS

4.1 PATIENT COHORT AND INCLUSION CRITERIA

A total of 39 non-Finnish South European patients who showed drug-induced or illicit substance-induced hyperthermia were treated by intensive care units and first aid departments, located throughout the national country. The Poison Control Center (PCC) and the National Center for Toxicological Information (CNIT) of the IRCCS ICS Maugeri of Pavia, was then engaged for advice to confirm the diagnosis of neuroleptic malignant syndrome (NMS). The PCC-CNIT unit applied the Guidelines of the International Expert Consensus for NMS diagnosis (Gurrera *et al.*, 2011, 2017) to enrol or exclude the patients in this study:

1. recent exposure to a trigger (dopamine antagonist or dopamine agonist withdrawal);
2. hyperthermia >38.0°C for more than 6 hours, resistant to classic pharmacological treatment (with antipyretics and NSAIDs);
3. muscle rigidity;
4. rhabdomyolysis with creatine kinase elevation (at least 3 times upper the normal limit [171 U/l for males, 145 U/l for females]);
5. signs of SNS lability (BP elevation, ≥ 25% baseline; BP fluctuation, ≥ 20 mmHg (diastolic) or ≥ 25 mmHg (systolic) change within 24 h);
6. signs of hypermetabolism (tachycardia [≥ 25% above baseline] and tachypnoea [≥ 50% above baseline];
7. negative workup for other aetiologies.

All recruited patients were enrolled accordingly to the ethics committee protocols.

4.2 PATIENT ENROLMENT

After the initial recruitment of 39 people, 7 individuals were excluded, because they did not completely satisfy the inclusion criteria described above:

- one sample belonged to a subject who had developed malignant hyperthermia (MH) subsequent the inhalation of anaesthetics (halogenated gases);
- the second sample belonged to a patient who developed serotonin syndrome (SS) following the intake of SSRI;
- the third subject was a Parkinson's disease patient which developed hyperthermia after the abrupt discontinuation of therapy with dopamine-agonist medications, but did not present muscle rigidity, and was therefore excluded;
- another subject was not included because he had no muscle rigidity;
- a fifth subject presented with muscle rigidity and hyperthermia following administration of long-acting injectable paliperidone palmitate, that resulted in high serum drug concentrations, but could not be classified as NMS and was therefore excluded;
- another subject, in addition to not having muscle rigidity, was not included because he did not develop NMS at all;
- the last excluded subject, even if he had received a depot antipsychotic drug, had not presented neither hyperthermia nor muscular rigidity.

The remaining 32 patients were grouped into two categories by physicians based on the triggers: first group was made by 19 patients who developed NMS symptoms triggered by assumption of antipsychotic drugs, while the second group of 13 people showed NMS-like symptoms upon abuse of illicit substances.

In the present study only the first group was further investigated, who developed the NMS symptoms triggered by antipsychotic therapy.

4.2.1 The cohort of NMS patients and triggers

On the NMS group of patients, further stringent criteria were applied such as diagnostic criteria and risk factors.

Polypharmacy, intended both as co-administration of antipsychotics with different types and categories of drugs and medications (e.g. psychotropic drugs, lithium, carbamazepine), and the simultaneous administration of more than one antipsychotic drugs, even of different categories (first and second- generation) (Perry and Wilborn, 2012; Tse *et al.*, 2015; Schneider *et al.*, 2018), the abrupt discontinuation of treatment with dopamine-agonists were also taken into account as risk factors. Thus, two patients were subsequently excluded from the study for the following reasons:

- a patient was under treatment with the antiepileptic drug Lamotrigine, although it acts as a dopamine D_2 receptor agonist inhibitor, it is not an antipsychotic;
- one other patient was excluded because took a massive dose of Clothiapine (overdose), therefore not a therapeutic dose of medication.

At the end of the enrolment process 17 patients passed the diagnostic and selection criteria previously described. Among the 11 patients who received a monotherapy treatment, 4 patients received a FGA drug (3 haloperidol and 1 zuclopenthixol), 5 subjects received a SGA drug (3 aripiprazole, 1 clozapine and 1 quetiapine). Two patients developed recurrent NMS episodes, upon treatments with four different consecutive antipsychotic monotherapies (3 SGAs and 1 FGA), or with single treatments with the FGA drug, haloperidol and with the SGA, quetiapine. The remaining 6 NMS patients developed the symptoms after concomitant administration of various antipsychotics (multitherapy): 4 patients were treated with a simultaneous combination of FGAs and SGAs medications, and 2 patients were treated with a SGA multitherapy (**Table 4.1**).

Monotherapy		
FGA	**SGA**	**FGA or SGA**
3 haloperidol	3 aripiprazole	1 risperidone, olanzapine, aripiprazole-promethazine
1 zuclopenthixol	1 clozapine	1 haloperidol, quetiapine
-	1 quetiapine	-
4	5	2
Tot 11		

Multitherapy		
FGA + FGA	**SGA + SGA**	**FGA + SGA**
-	1 Clothiapine, clozapine, aripiprazole	1 Haloperidol, clotiapine
-	1 Quetiapine, aripiprazole	1 promazine, haloperidol, quetiapine
-	-	1 haloperidol, quetiapine , risperidone
-	-	1 Promazine, aripiprazole
-	2	4
Tot 6		

Table 4.1: Pharmacological treatment of NMS patients. The tables summarize the distribution of different FGAs or SGAs drugs, in monotherapy (on the right) and in multitherapy (on the left) among NMS patients.

Thus, SGAs were the most predominant drugs, being administered in 13 NMS subjects, in 5 cases alone (monotherapy), in 2 cases administered as monotherapy alternating with FGAs, and in 6 cases administered in multitherapy; while FGAs were administered in 10 patients, in 4 cases alone (monotherapy), in 2 cases as monotherapy alternating with SGAs and in 4 cases were administered in multitherapy with SGAs. No case of a patient being treated with more than one FGAs has been reported in this study.

4.2.2 The cohort of NMS patients and risk factors

A relevant risk factor encountered among NMS patients is the first intake or sudden variation of the treatment plan in a fairly short period of time before the onset of the syndrome (Mann *et al.*, 2003). In this cohort of patients, the onset of the NMS syndrome was reported within 30 days after the variation of the therapy in 9 out of 17 patients: for 5 patients a new antipsychotic was added to the therapeutic plan, in one patient the lithium treatment was suspended, in one subject the dosage of the drug was increased, on the contrary in another subject, the therapy was suspended and then resumed with a different dosage of drug. In addition, one patient experienced NMS at the first intake of antipsychotics.

Moreover, taking into account other risk factors such as environmental parameters and comorbidities, it was observed that in this cohort the onset of NMS occurred in 4 subjects in

conjunction with the raising of the environmental temperature, whereas 2 individuals were affected by other pathologies (comorbidities).

4.2.3 Age and gender distribution

Age and gender distribution among the patient and control groups analyzed was evaluated.

The patient group consisted of 17 individuals in total, including 10 males and 7 females (mean age 47.4 years, median 49 years, SD 17.27). Meanwhile, the control group consisted of 70 generally healthy individuals, including 20 males and 49 females (mean age 42 years, median 42 years, SD 15.5). Age and gender of a control individual were not known.

The gender distribution among the patients and controls groups is detailed below (**Figure 4.1**). A significant imbalance in the distribution of gender was observed among patients and controls through an analysis with a Pearson's chi-squared test ($X_1^2 = 4{,}46$, $p = 0.035$).

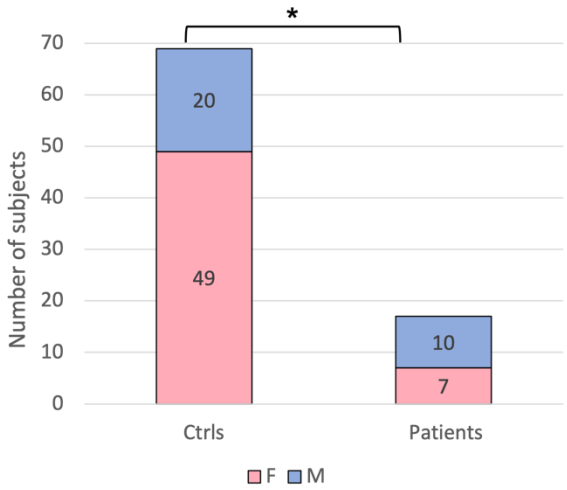

Figure 4.1: Gender distribution between patients and controls. Distribution of male (in light blue) and female (in pink) between controls and patients is detailed. Person's chi-squared test significance is reported with an asterisk ($^{*}p < 0.05$).

The distribution of age revealed no significant difference at diagnosis among patients with drug-induced hyperthermia and controls using a Student's two-sample *t*-test with equal variances ($t_{84} = -0.5241$, $p = 0.69$).

4.3 *DRD2* POLYMORPHISM ANALYSIS

Previous reports on asiatic populations described conflicting results on possible association of three polymorphisms with NMS: the rs1800497, the rs1799732 and the rs1799978 variant associated with the *DRD2* gene (**Figure 4.2**).

In our previous study (Veronica Cattaneo, PhD thesis 2021), these SNVs were investigated for possible association with NMS in a south european cohort of 25 patients, among which antypsychotic drugs were the triggers of NMS in 12 patients, whereas illicit substances were the triggers for the remaining 13 patients who showed NMS-like symptoms.

In this PhD thesis, the cohort was extended to a total of 17 patients affected by NMS triggered by antipsychotic treatment and the above SNPs were again investigated. A total of 70 healthy individuals were also investigated as a control population.

To test for potential bias due to enrollment processes, i.e., founder effect or genetic isolation, the allelic frequencies of the three genetic variants, rs1800497, rs1799732, and rs1799978 were calculated among the 70 control individuals. The frequencies of the investigated SNPs was found in the range reported by the gnomAD (https://gnomAD.broadinstitute.org/) for southern European population, in agreement with the ethnic distribution of the population residing in this geographic area, and showed no significant deviation from Hardy-Weinberg equilibrium for rs1800497 and rs1799732 ($p>0.05$), whereas it differed slightly for rs1799978 ($p=0.04$).

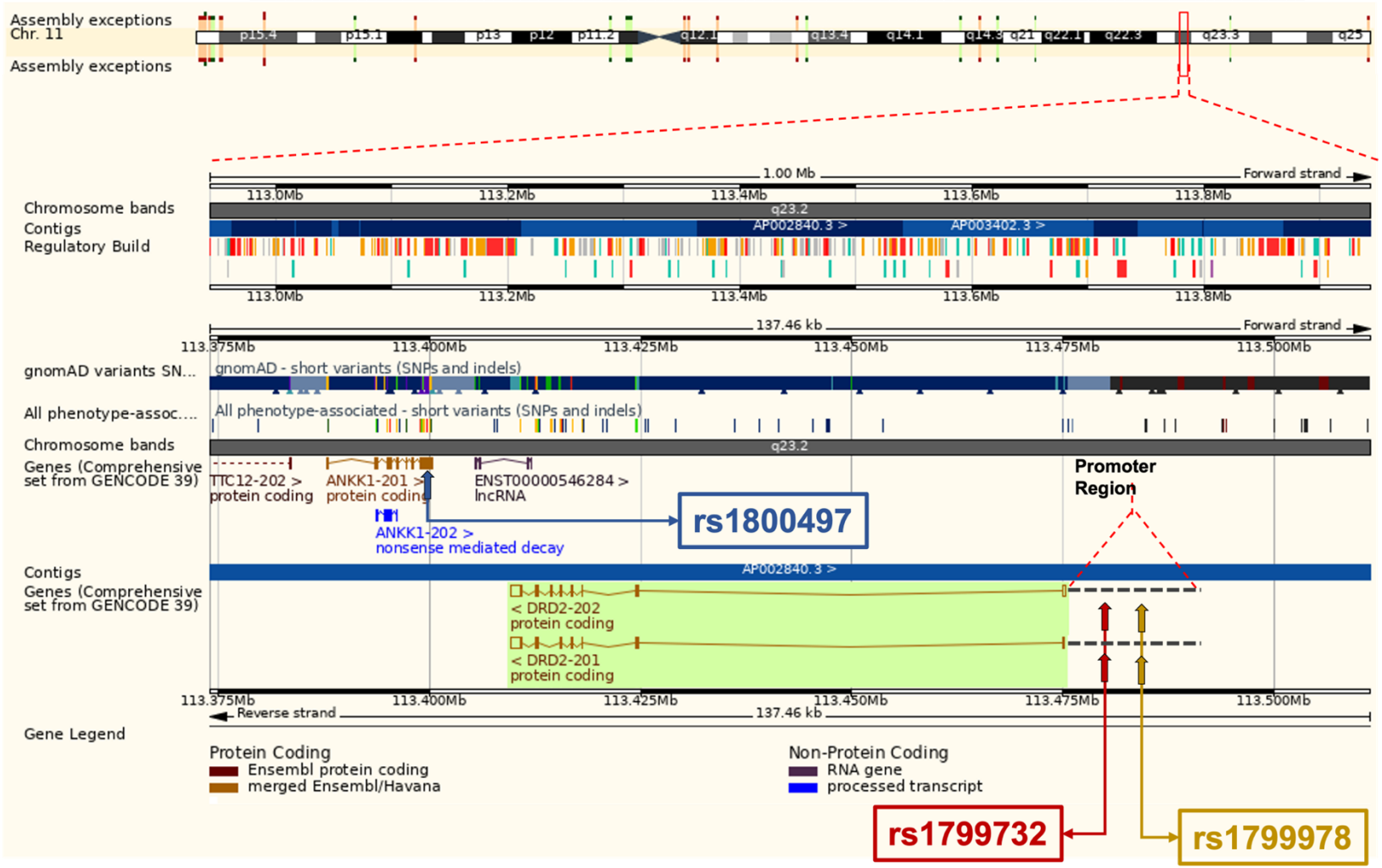

Figure 4.2: Human DRD2 and ANKK1 genomic context. From the top, a schematic representation of human chromosome 11 is reported. The cytogenetic position of DRD2 gene is indicated by a red bar. The detail of the corresponding genomic region from Ensembl Homo sapiens version 105 (GRCh38.p13) is reported at the bottom. ANKK1 gene is represented on the forward strand. DRD2 gene is reported on the reverse strand. Filled boxes are coding exons and empty boxes are untranslated exons, while introns are indicated by lines connecting boxes. The genetic variants rs1800497 (in blue) located within exon 8 of ANKK1 gene, rs1799732 (in red) and rs179978 (in yellow) located within promoter region of DRD2 gene, are boxed and their position is indicated by arrows.

4.3.1 The *ANKK1* variant rs1800497

The rs1800497, also known as Taq1A or TaqIA, is a human variant located whithin the exon 8 of the *ANKK1* gene, located about 10kb at the 5' side of the *DRD2* gene according to the Reference sequence of the human genome (GRCh38.p13). It is classified as a missense variant, mapping at position 11:113400106 and characterized by a G to A nucleotide change (forward strand, Ensembl Release 105, GRCh38.p13) resulting in a glu-to-lys substitution within the eleventh ankyrin repeat of *ANKK1*.

PCR with *NM1F* and *NM1R* primers was carried out to amplify a 236 bp PCR amplicon on human genomic DNA. Both Taqα1 RFLP analysis and Sanger sequencing were applied to investigate this SNP on a total of 17 patients and 70 controls (**Figure 4.3**).

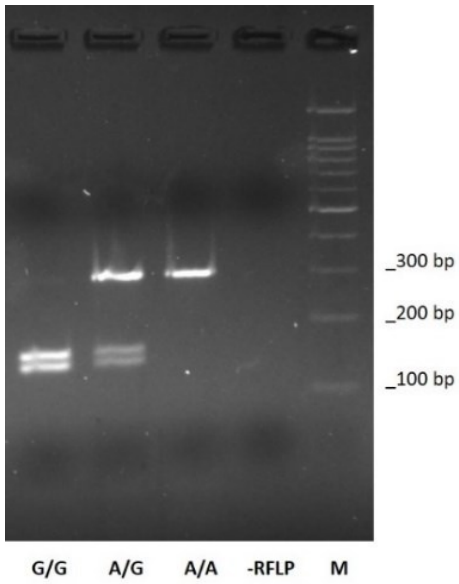

Figure 4.3: PCR-RFLP for rs1800497. The 236 bp fragments amplified by PCR with primers *NM1F* and *NM1R* were digested with TaqαI enzyme. The sizes of the digestion fragments resolved on a 3% agarose-TBE gel for rs1800497 genotypes were: A/A 236 bp (uncleaved); A/G 236, 125, 111 bp; G/G 125, 111 bp. Lane M represents a 100 bp DNA ladder.

Among the 70 healthy individuals of the control population, 2 subjects had the genotype A/A, 17 had the genotype A/G and 51 individuals had the genotype G/G, corresponding to a frequency of 0.15 for allele A and 0.85 for allele G. Accordingly, the frequency of the A allele is in the expected range of the GnomAD database.

About the NMS group, a total of 12 patients had the homozygotic G/G genotype whereas 4 had the heterozygotic genotype A/G and 1 had the homozygotic genotype A/A, corresponding to a frequency of 0.177 for allele A and 0.823 for allele G. Therefore, a slight increase of A-allele

frequency over the expected frequency was observed among the patients in comparison to controls (f=0.150) (**Figure 4.4**).

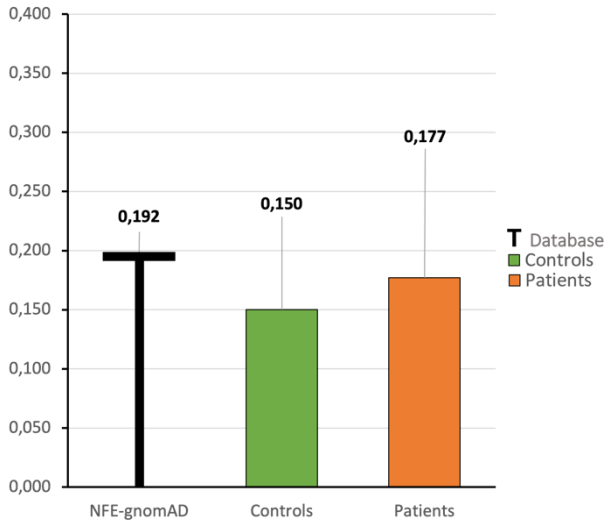

Figure 4.4: Minor allele frequency of rs1800497 variant of ANKK1 gene. The bar plot shows the frequency distribution of the minor allele A of the variant rs1800497 between patients (in orange) and controls (in green). The frequency of allele A for the Non-Finnish European population according to gnomAD database (v2.1.1) is also reported (in black).

Thus, a dominant genetic model was built to infer possible genetic association of the specific risk allele A with the NMS outcome, thus the (AA + AG) genotypes *vs* homozygotic GG genotype of rs1800497 SNP frequencies were investigated, however no significant increase of the A allele was found in the NMS patients compare to healthy individuals. Although slightly lower than the allele frequency reported in the database GnomAD for the non-Finnish European population, this fluctuation, likely due to the size of the cohort, is in the expected range for the Southern European population according to GnomAD database (**Table 4.2**).

rs1800497				
	NMS patients ($n = 17$)		Controls ($n = 70$)	
	N	frequency	N	frequency
G/G	12	0.705	51	0.729
A/G	4	0.235	17	0.243
A/A	1	0.006	2	0.029
A/A + A/G	5	0.294	19	0.129

Table 4.2: Frequency distribution of the rs1800497 variant. The genotypic distribution of the rs1800497 variant of ANKK1 gene among NMS patients and controls is detailed.

4.3.2 The *DRD2* promoter variant rs1799978

The rs1799978 is mapped at the 3' side of the DRD2 locus at position GRCh38p13 11:113475629 (forward strand), since the DRD2 gene is encoded by the lower strand this polymorphism maps within the promoter region of the human *DRD2* gene, at about 231bp from the Transcription Start Site (TSS) of the gene. This SNP was historically investigated by a RFLP (restriction fragment length polymorphism) with MaeIII restriction enzyme (Arinami *et al.*, 1997) (**Figure 4.5**).

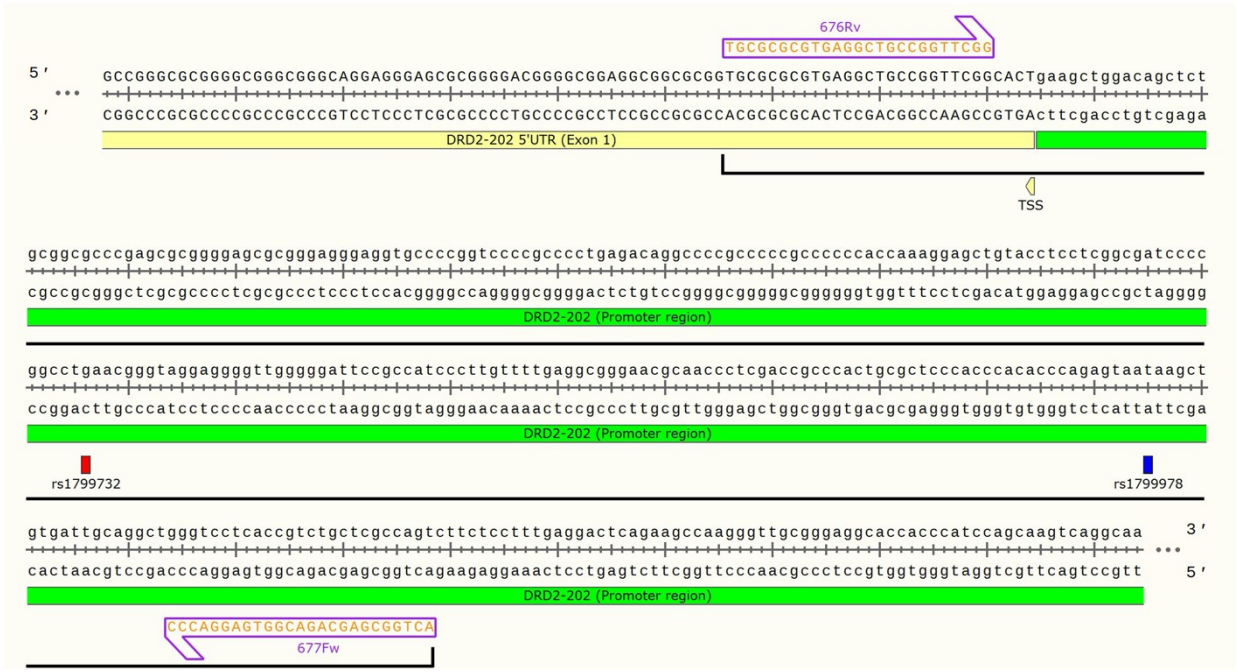

Figure 4.5: Nucleotide sequence of the promoter region of *DRD2*. In figure is detailed the nucleotide sequence of the *DRD2* promoter region, amplified by PCR. Primers (name and sequence) used for PCR are reported by arrows. The variant rs1799732 and rs1799978 are indicated by a red and blue box, respectevely. Abbreviations: TSS, transcription start site; Fw, forward; Rv, reverse.

PCR with *D2-677Fw* and *D2-676Rv* primer set was carried out to amplify a 303 bp amplicon on human genomic DNA of the 17 NMS patients and 70 healthy individuals. The amplicon was then digested with MaeIII restriction enzyme, the restriction fragment length polymorphism (RFLP) resolved on TBE-agarose gel electrophoresis and/or Sanger sequenced (**Figure 4.6**). According to gnomAD genomes v2.1.1, the expected MAF (minor allele frequency) of the C allele is 0.12, the highest MAF detected in any population is 0.28 whereas the MAF of the non-Finnish European population (NFE) is 0.055.

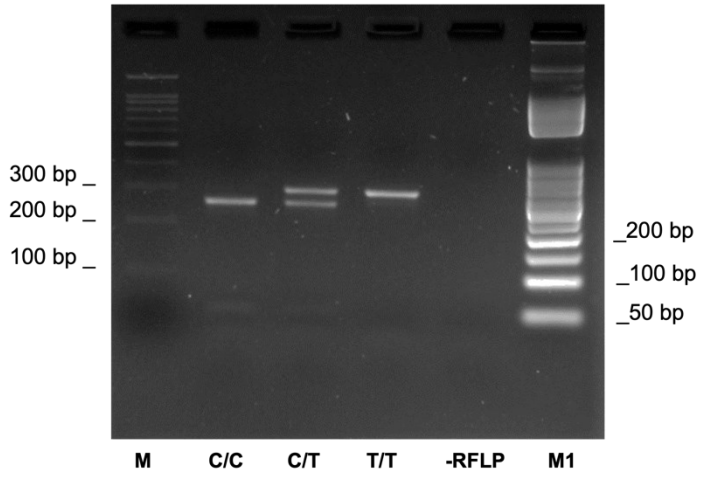

Figure 4.6: PCR-RFLP of rs1799978 variant. The 303bp fragments of the DRD2 promoter region amplified by PCR with primers D2-677Fw and D2-676Rv were digested with MaeIII enzyme. The 303bp amplicon remained uncleaved when the reference allele T was present, whereas it was cut in 260 bp and 43 bp fragments in presence of the the C allele. The sizes of the digestion fragments resolved on a 3% agarose-TBE gel and visualized under UV-light after ethidium bromide staining were (from left to right): C/C 260, 43 bp; C/T 303, 260, 43 bp; T/T 303 bp (uncleaved);. Lane M and M1 represent a 100 bp and a 50 bp DNA ladder, respectively.

Among the 70 control individuals, 64 had the T/T genotype, 5 the C/T genotype and 1 C/C genotype, respectively. Thus, the allelic frequencies for the healthy population of the cohort enrolled in this study were 0.95 for the allele T and 0.05 for the variant allele C, as expected for the European non-Finnish population (gnomAD database f= 0.06). All the 17 NMS patients had the T/T genotype, corresponding to an allelic frequency of 1.00 for the allele T. Thus, although slightly decreased, the values regarding the C allele frequency appear in the same range described by the GnomAD database (**Table4.3**). Because the rare allele was not present in the patient group, no dominant genetic model was built to infer possible genetic association with the rare allele C.

rs1799978				
	NMS patients ($n = 17$)		Controls ($n = 70$)	
	N	frequency	N	frequency
T/T	17	1.00	64	0.914
C/T	0	0.00	5	0.072
C/C	0	0.00	1	0.014
C/C + C/T	0	0.00	6	0.088

Table 4.3: Frequency distribution of the rs1799978 variant. The genotypic distribution of the rs1799078 variant of *DRD2* gene among NMS patients and controls is detailed.

4.3.3 The *DRD2* promoter variant rs1799732

The rs1799732 is mapped on the 3' side of the *DRD2* locus of the human genome (GRCh38.p13), whose gene is encoded by the lower strand. This SNP is an INDEL variant, indeed the reference allele G at position chr11:113475530 (forward strand) changes to GG allele by an insertion of G nucleotide (Ensembl Release 105, GRCh38.p13). This variant was historically named -141C Ins/Del since it mapped on the lower strand (reverse strand) at 141 bp from the transcription start site (TSS) of the *DRD2* regulatory region, (https://www.ensembl.org/Homo_sapiens/Variation/Explore?r=11:113475030-113476030;v=rs1799732;vdb=variation;vf=165588878). The most recent revision of the human reference genome sequence (GRCh38.p13) mapped this variant at 131bp upstream of the TSS of the *DRD2*.

According to the Ref-seq SNP (reference SNP) the G allele is the ancestral allele and the insertion variant is GG. The MAF of the G allele according to the 1000 Genomes Phase 3 combined population is 0.24 (G), and according to GnomAD the non-Finnish European population (NFE) the G allele frequency is 0.097, whereas the GG allele is 0.903. The G insertion (GG variant) generates the target site for the BstNI restriction enzyme, thus the promoter region of *DRD2* was PCR amplified with primer set *D2-677Fw* and *D2-676Rv* to generate a 303 bp DNA fragment and the presence of the GG variant detected by BstNI Restriction Fragment Length Polymorphism (**Figure 4.7**). Genotypes were also confirmed by Sanger sequencing.

The RFLP samples, digested with the BstNI enzyme (NEB, New England Biolabs), were resolved on a 3% agarose-TBE gel electrophoresis to observe the band of 303bp of the G allele or the two fragments of 159 bp and 144 bp derived by the digestion of the GG allele.

The G insertion (GG allele) was also confirmed by Sanger sequencing as shown in the electropherograms (**Figure 4.8**).

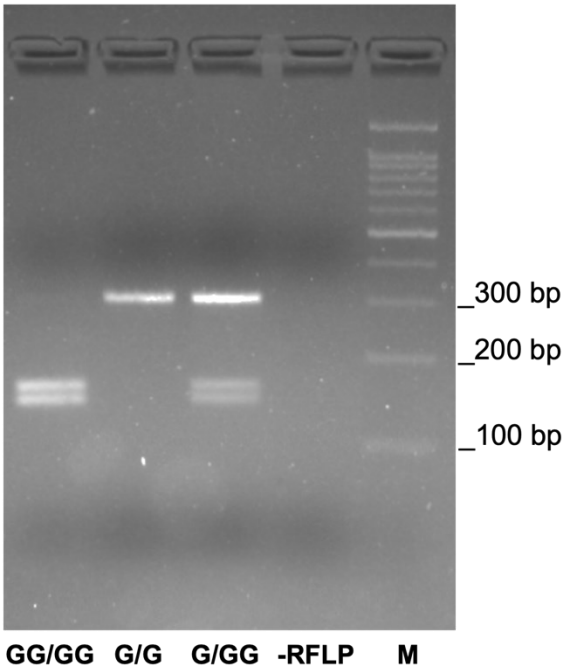

Figure 4.7: PCR-RFLP for rs1799732 variant. The 303bp fragments of the DRD2 promoter region amplified by PCR with primers D2-677Fw and D2-676Rv were digested with BstNI enzyme. The sizes of the digestion fragments resolved on a 3% agarose-TBE gel and visualized under UV-light after ethidium bromide staining were (from left to right): GG/GG 159, 144 bp, G/G 303 bp (uncut), G/GG 303, 159, 144 bp. The negative of reaction is -RFLP and lane M represents 100 bp DNA ladder.

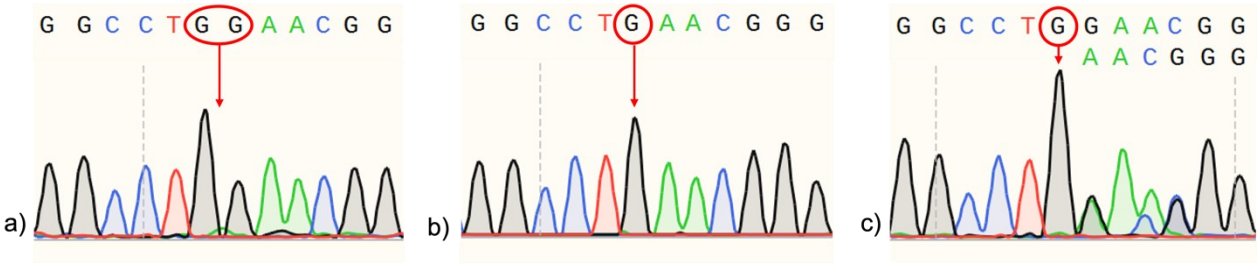

Figure 4.8: Electropherograms of PCR-fragments. In the figures are detailed the three possible electropherograms obtained after Sanger's sequencing analysis. **a)** the homozygous GG/GG, **b)** the homozygous G/G, and **c)** the heterozygous G/GG genotypes.

The genotype frequencies among the healthy individuals was 0.87 for the GG/GG genotype (found in 61 subjects), 0.11 for the G/GG genotype (found in 8 subjects), and 0.014 for the G/G genotype (found in 1 subject). Thus, the allele frequency of the healthy individuals was 0.929 for the GG variant allele, and 0.071 for the G reference allele, in agreement with the allele frequency reported in GnomAD database for south European non finish population. For statistical analysis,

subjects carrying at least one copy of the less frequent allele G (homozygotic G/G and heterozygotic G/GG genotypes) were grouped together and named G-carriers (homozygotic G/G and heterozygotic G/GG genotypes), whereas the individuals carrying the homozygotic GG/GG genotype were named G-non-carriers.

To investigate possible statistic association of this SNP variant (genetic risk) and the risk of developing NMS upon antipsychotic treatment and exclude confounders or effect modifiers, statistical analysis was performed. No significant distributional difference of gender was found among G-carriers and G-non-carrier subjects, on a Pearson chi-square test (X_1^2 = 0.079, p = 0.779). No difference about age distribution was found between G-non carrier individuals (mean age 46.39 years, SD 14.47) and G- carrier individuals (mean age 42.19 years, SD 15.51) on a Student's two-sample *t*-test with equal variances (t_{84}= 1.0337, p= 0.1521). Thus, gender and age are not confounders or effect modifiers of the association between the syndrome and genetic risk factor, for people carrying the rs1799732 variant.

Among the 17 patients, the homozygotic GG/GG genotype was found in 10 patients, the G/GG genotype in 7 patients and no patient with homozygotic G/G genotype was found. Thus, the genotype frequency of GG/GG patients was f=0.59 and f=0.41 for the G/GG genotype, respectively. Therefore, the allele frequency among the NMS patients enrolled in this study was 0.929 for the GG (insertion variant) allele, and 0.21 for the G reference allele.

Thus, the G allele frequency increased approximately 3.2-fold over controls in NMS patients on the Fisher's exact 1-sided test (p=0.013) (**Table 4.4, Figure 4.9**).

rs1799732					
	NMS patients ($n = 17$)		Controls ($n = 70$)		$p - value$
	N	frequency	N	frequency	
GG/GG	10	0.588	61	0.871	
G/GG	7	0.412	8	0.114	
G/G	0	0.000	1	0.014	
G/G + G/GG	7	0.412	9	0.129	*0.013

Table 4.4: Frequency distribution of the rs1799732 variant. The genotypic distribution of the rs1799732 variant of DRD2 gene among NMS patients and controls is detailed. Genetic association was performed considering G/G + G/GG genotypes versus GG/GG genotypes.

With the aim to estimate the genetic susceptibility of developing NMS among G-carrier patients who developed NMS after antipsychotic administration, logistic regression model was

performed. The results indicated that patients with G/G or G/GG genotype (G-carriers) had a 5.5 times greater risk of developing NMS than those with GG /GG genotype (G-non-carriers) controlling for age and sex (OR = 5.476, 95% CI = 1.499–19.997; p = 0.010).

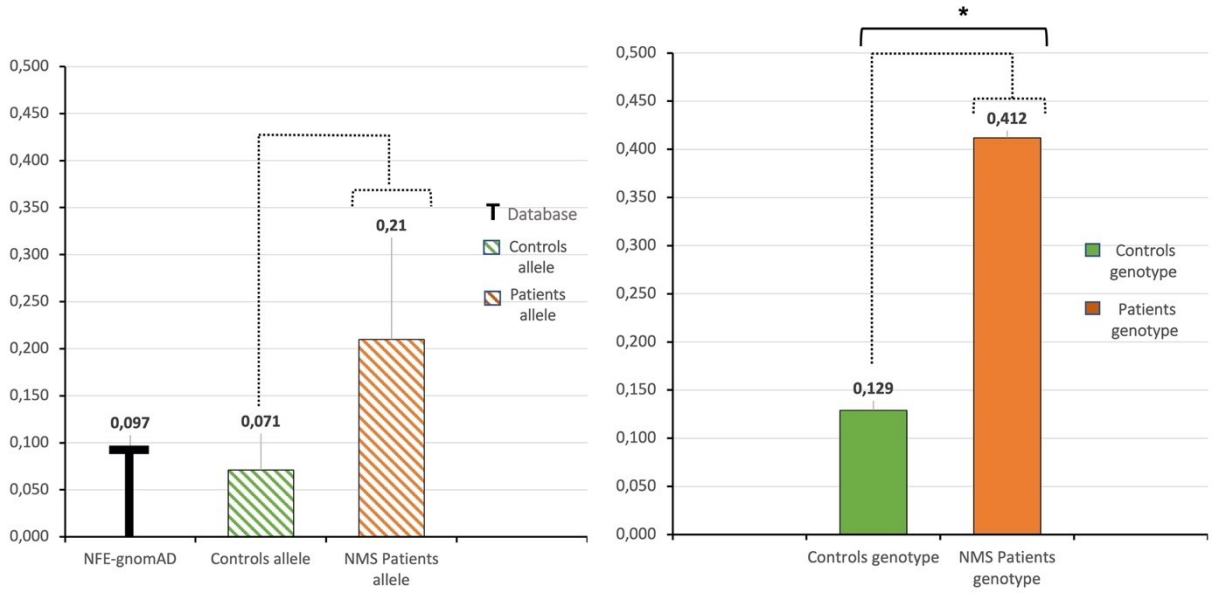

Figure 4.9: Frequency distribution of G- allele and genotype. The bar plot on the left details the frequency distribution of the G- allele of the variant rs1799732 between NMS (orange line bar) controls (green line bar) and gnomAD database for the European population (black line). The bar plot on the right details the frequency distribution of the G-carriers (G/G and G/GG) of the variant rs1799732 between NMS patients (orange bar) controls (green bar) The distributional difference of the G-carrier between controls and NMS patients was statistically significant on Fisher's exact test (*p< 0.05).

It is noteworthy that SGAs (second-generation antipsychotics) were administered in 6 of the 7 NMS patients with the G/GG genotype (85.5%). Of these, 4 patients received a monotherapy treatment with SGA (3 individuals with aripiprazole only and 1 with quetiapine); whereas one patient was treated with SGAs and FGAs taken concomitantly, and the other developed 4 distinct NMS episodes after consecutive single treatments of SGAs or FGAs administration. With regard to first-generation antipsychotics, however, they were administered in only 3 of the 7 G-carrier patients (42.8%); in particular, two subjects (just described) experienced NMS after treatment with different APs (FGAs and SGAs) taken concomitantly or after administration of SGAs or FGAs administered singly, whereas just one patient developed the syndrome after taking only one FGA (zuclopenthixol). Overall, second-generation antipsychotics are the triggers in 13 out of 17 NMS (76.5%) patients, whereas first-generation antipsychotics were detected in 10 of 17 NMS patients (58.8%) (**Table 4.5**).

ID	Genotype	Antipsychotics				Notes
MP4	G/GG	Aripiprazole	-	-	-	Monotherapy
MP12	G/GG	Aripiprazole	-	-	-	Monotherapy
MP18	G/GG	Aripiprazole	-	-	-	Monotherapy
MP21	G/GG	Risperidone	Promethazine	Olanzapine	Aripiprazole	4 distinct treatment
MP25	G/GG	Quetiapine	Haloperidol	Risperidone	-	Multitherapy
MP29	G/GG	Quetiapine	-	-	-	Monotherapy
MP33	G/GG	Zuclopenthixol	-	-	-	Monotherapy
MP6	GG/GG	Clothiapine	Clozapine	Aripiprazole	-	Multitherapy
MP7	GG/GG	Haloperidol	Clothiapine	-	-	Multitherapy
MP16	GG/GG	Haloperidol	-	-	-	Monotherapy
MP20	GG/GG	Quetiapine	Aripiprazole	-	-	Multitherapy
MP22	GG/GG	Quetiapine	Promazine	Haloperidol	-	Multitherapy
MP23	GG/GG	Clozapine	-	-	-	Monotherapy
MP24	GG/GG	Haloperidol	Quetiapine	-	-	2 distinct treatment
MP32	GG/GG	Haloperidol	-	-	-	Monotherapy
MP35	GG/GG	Haloperidol		-	-	Monotherapy
MP37	GG/GG	Promazine	Aripiprazole	-	-	Multitherapy

Table 4.5: Genotypic and Pharmacological distribution among NMS patients. In table is detailed the genotypic distribution of the rs1799732 variant of DRD2 gene among 17 NMS patients, their therapy with FGAs (in light blue) or SGAs (in light green) and the type of therapy.

Although these results are not supported by statistical tests, mainly due to too small numbers, it is however interesting to note that SGAs are the most frequent trigger element in our NMS patient's cohort, and they also have an even doubled frequency in G-carrier subjects.

In conclusion, the rs1799732 genetic variant may be considered a putative pharmacogenetic biomarker associated with an increased risk of developing NMS following administration of antipsychotic therapy. Individuals carrying at least one G allele (G/GG or G/G genotypes) are predisposed to a 5.5-fold increased risk of developing NMS compared with non-carriers. This evidence, validated by statistical calculations, supports the hypothesis that there is a genetical predisposition to susceptibility to the development of neuroleptic malignant syndrome. Moreover, although it is not yet possible to statistically validate this finding due to the low number of subjects, it would seem that patients carrying the G allele are at higher risk of developing SNM following the intake of a second-generation antipsychotic than a first-generation one.

4.4 WHOLE GENOME SEQUENCING

The aforementioned results supported the hypothesis that genetic traits may contribute to NMS susceptibility.

To deeply investigate this hypothesis, the gDNAs extracted from peripheral venous blood cells of 11 selected NMS patients were processed for whole genome sequencing at 30X coverage.

The results described in this PhD thesis were then focused on discovering shared CNVs (Copy Number Variation) and/or SVs (Structural Variants) among the patients that could be associated with NMS predisposition.

4.4.1 Selection of patients for WGS

Among the patients with NMS diagnosis triggered by antipsychotics, a total of 11 patients were selected for WGS analysis by applying even more stringent criteria to minimize parameters within the cohort, such as the trigger and the treatment mono- vs multi- therapy. Thus, NMS patients were sub-grouped in those (mono FGA: 4 patients) who received a monotherapy of FGA and those (mono SGA: 5 patients) who were treated with a SGA monotherapy. In addition, 2 patients were treated in two consecutive period with only one antipsychotic a time, FGA or SGA therapy (mono FGA + mono SGA: 2 patients).

Notably, the haloperidol was prescribed in 4 out of 6 patients who received at least one FGA, and the aripiprazole was prescribed in 4 out 7 patients who received at least one SGA (**Table 4.1**). Among the 11 selected patients, 6 individuals treated with a SGA monotherapy were G-carriers (genotype G-/GG) for the -141C Ins/Del genetic variant mapped on the *DRD2* promoter region (rs1799732), whereas 5 individuals treated with a FGA monotherapy were G-non carriers (genotype GG/GG) (**Table 4.10**).

The 11 selected NMS subjects were all of non-Finnish European ethnicity, of which 5 were female and 7 were male.

The remaining 6 of the 17 NMS patients who received a multitherapy, a cocktail of FGA, SGA and/or other drugs (antidepressants, lithium, anticonvulsants, or drugs used in the treatment of Alzheimer's) were not included in this WGS analysis (**Table 4.6**).

Genotypic and Pharmacological Distribution			
	G-/GG	GG/GG	Tot
FGA (Monotherapy)	1	3	4
SGA (Monotherapy)	4	1	5
FGA or SGA (Monotherapy)	1	1	2
FGA + SGA (Multitherapy)	1	3	4
SGA+ SGA (Multitherapy)	-	2	2
Tot	7	10	17

Table 4.6: Genotypic and Pharmacological distribution. In table is detailed the genotypic distribution of the rs1799732 variant of DRD2 gene among NMS patients, and their therapy with FGAs or SGAs medications.

4.4.2 Quality Check report and analysis results

The Illumina platform generates raw images and base calling with an integrated primary analysis software, called RTA (real time analysis). An initial step of this pipeline is the generation of FastQC files that allows an immediate outlook of the high-throughput sequencing quality. The FastQC data files are intended to provide a simple set of analyses and a quality check (QC) report that can identify issues originated during the massive sequencing or preparation of the sequencing library (**Figure 4.10**).

To assess the quality of sequencing, the statistical parameters to be evaluated are fastq statistics, allignment and coverage statistics, the fragment sizes and the depth of coverage.

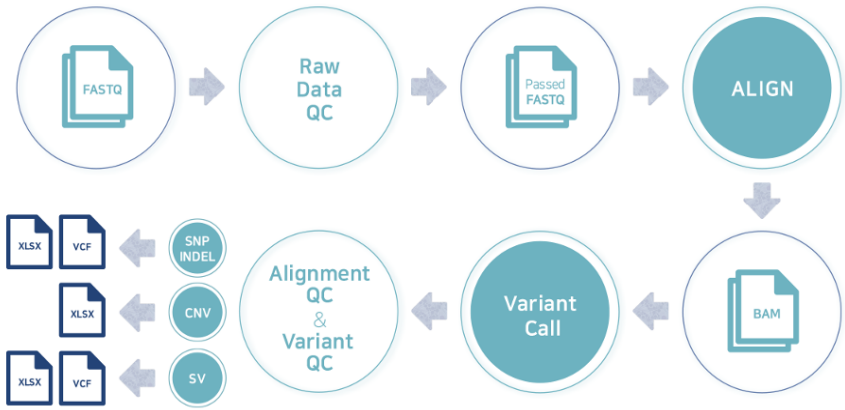

Figure 4.10: Schematic representation of data processing. The figure schematically summarizes the basic steps involved in data processing after sequencing analysis.

4.4.2.1 Fastq Statistics

The fastq statistics provide an immediate overview of the quality of sequencing, for each sample analyzed, according to the total amount of bases sequenced, the total number of reads, the percentage GC content, and the phred quality score (**Figure 4.11-4.12**).

The sequencing quality score of a given base, Q, is defined by the following equation:

$Q = -10\log_{10}(e)$

where e is the estimated probability of the base call being wrong.

- Higher Q scores indicate a smaller probability of error.
- Lower Q scores can result in a significant portion of the reads being unusable. They may also lead to increased false-positive variant calls, resulting in inaccurate conclusions.

A quality score of 20 (Q20) represents an error rate of 1 in 100 (meaning every 100 bp sequencing read may contain an error), with a corresponding call accuracy of 99%.

When sequencing quality reaches Q30, virtually all of the reads will be perfect, with no errors or ambiguities. This is why Q30 is considered a benchmark for quality in next-generation sequencing (NGS) (https://emea.illumina.com/science/technology/next-generation-sequencing/plan-experiments/quality-scores.html).

The average quality for each sequencing run (or cycle) R1 and R2, for each sample analyzed, is displayed in the graphic below (**Figure 4.13**). Along the x-axis of this graph, all the individual bases for each reading analyzed are reported, and for each base call the distribution of values is plotted. The blue line of quality scores should remains high throughout the run, or at least above 20, for good quality run and basic call. In our samples, the blue line never fell below 30, thus determining very good quality sequencing. Detailed graphs for each of the 11 patients analyzed are provided in the Appendix.

The fastq format, which has become the standard for storing results from high-throughput sequencing tools such as the Illumina platform, is an ASCII-code that determines the quality of the nucleotide sequence after sequencing in phred score format.

In our samples, the mean values of fast Q20 and fast Q30 were 96.7% and 91.7%, respectively, resulting in very high sequencing quality, with almost no sequencing errors (**Figure 4.11, Table 4.7**). The GC content of each patient is also in the expected range and comparable to the reference sequence.

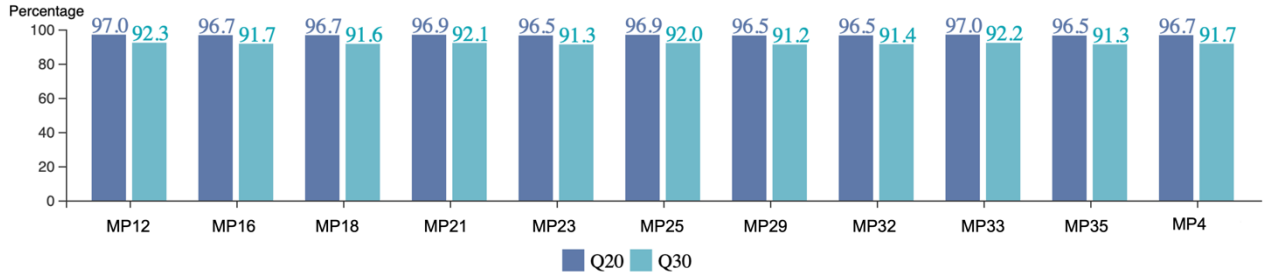

Figure 4.11: Q20/Q30 scores of Raw data. The bar graphic illustrates in detail the percentage of Q20/Q30 for each sample.

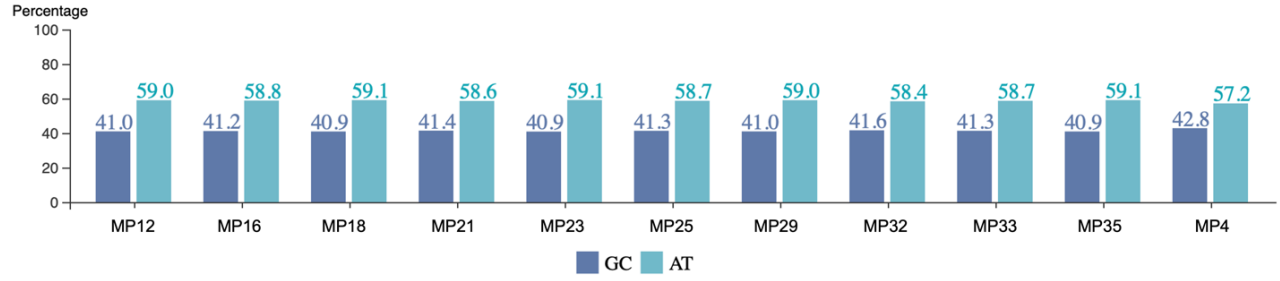

Figure 4.12: GC content Raw data. The bar graphic illustrates in detail the GC % content and AT % content among each sample.

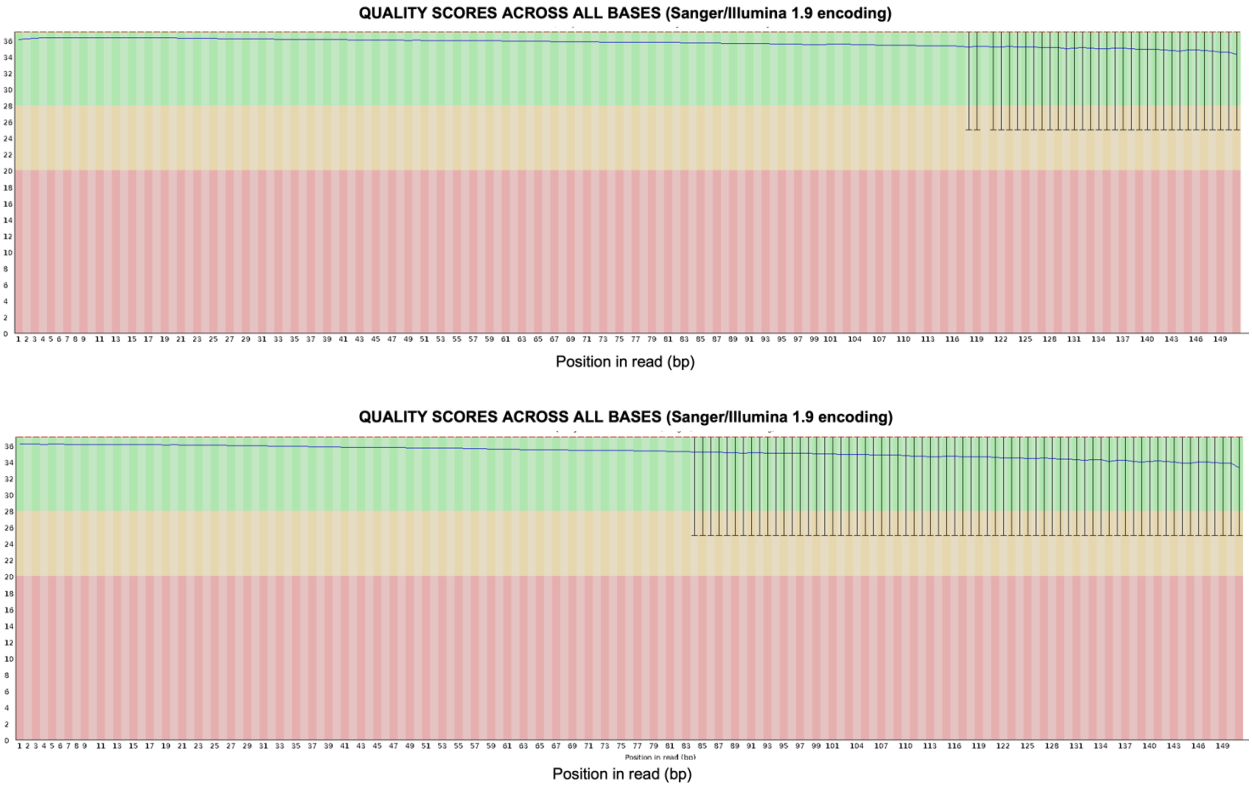

Figure 4.13: Quality by cycle. The graphic displays an example of the per base sequencing quality scheme for each cycle run (R1 and R2). The blue line represents the mean quality scores.

4.4.2.2 Alignment statistics and coverage

Additional parameters to be evaluated to define sequencing quality include the amount of total reads and total read length, the size of the fragment for each read and the depth of coverage (pre- and post-alignment statistics) (**Table 4.7, Table 4.8**).

The length of the fragments size analyzed during whole genome sequencing for each read was reported to have an average length of about 420 bp, with a standard deviation of about 98 (**Table 4.9, Figure 4.15**), while the length of each read was 151 bp, which multiplied by the total number of reads gives the total length of each patient's genome in base pairs (**Table 4.10**, **4.13**).

Another important parameter to evaluate for the goodness of sequencing is the depth of coverage, which represent the number of times a nucleotide is read during sequencing. Greater depth of coverage increase confidence in the final results and helps distinguish sequencing errors from single nucleotide polymorphisms. The depth of coverage from 1X to 30X is reported (**Table 4.8, Figure 4.14**); as can be seen, as the sequencing depth increases the reference values decrease in inverse ratio.

For each of the 11 sequenced patients, an overview report has been provided, the results of which are specified below (**Table 4.10**). The data of sequencing parameters, evaluated together or individually, show not only that from a technical point of view, the average coverage and depth of sequencing are comparable and overlapping among all samples analyzed, but also that the quality of sequencing was of excellent quality for each subject analyzed. Low Q scores can increase false-positive variant calls, which can result in inaccurate data. Our samples, however, were all sequenced with a phred 30 score >91% for all individuals, so the identified variants are not due to sequencing biases. Therefore, after these assessments, CNV (Copy Number Variation) and/or SV (Structural Variants) shared among patients that might be associated with susceptibility to NMS were analyzed.

Sample ID	% > Fastq Q20	% > Fastq Q30	Total reads	Read length (bp)	Total yield (Mbp)	Reference size (Mbp)	Throughput mean depth (X)
MP4	96,7	91,7	776.130.614	151,0	117.195	2.934	39,9
MP12	97,0	92,3	788.172.282	151,0	119.014	2.934	40,6
MP16	96,7	91,7	777.378.116	151,0	117.384	2.934	40,0
MP18	96,7	91,6	804.530.302	151,0	121.484	2.934	41,4
MP21	96,9	92,1	749.939.132	151,0	113.240	2.934	38,6
MP23	96,5	91,3	794.185.514	151,0	119.922	2.934	40,9
MP25	96,9	92,0	771.190.720	151,0	116.449	2.934	39,7
MP29	96,5	91,2	784.172.170	151,0	118.409	2.934	40,3
MP32	96,5	91,4	773.234.134	151,0	116.758	2.934	39,8
MP33	97,0	92,2	739.118.110	151,0	111.606	2.934	38,0
MP35	96,5	91,3	736.069.052	151,0	111.146	2.934	37,9

Table 4.7: Pre-alignment statistic. The table shows the pre-alignment statistic data for each patient. The analysis data is quite similar and overlapping among patients.

Sample ID	% >= 1X coverage	% >= 5X coverage	% >= 10X coverage	% >= 15X coverage	% >= 20X coverage	% >= 30X coverage
MP4	99,1	98,7	98,3	96,6	88,2	46,6
MP12	99,8	99,5	98,7	96,0	91,3	53,9
MP16	99,8	99,4	98,8	96,3	91,8	57,4
MP18	99,1	98,7	98,3	97,8	95,8	62,6
MP21	99,8	99,3	98,5	95,7	90,4	51,0
MP23	99,8	99,4	98,8	96,6	92,7	65,1
MP25	99,8	99,4	98,7	96,1	91,5	56,0
MP29	99,8	99,4	98,8	96,6	92,5	62,6
MP32	99,0	98,6	98,3	97,6	94,3	55,5
MP33	99,0	98,6	98,3	97,4	92,5	44,3
MP35	99,1	98,7	98,3	97,5	93,5	46,7

Table 4.8: Alignment Coverage. The table shows the alignment statistic data for each patient. The reference values decrease in inverse ratio as sequencing depth increases

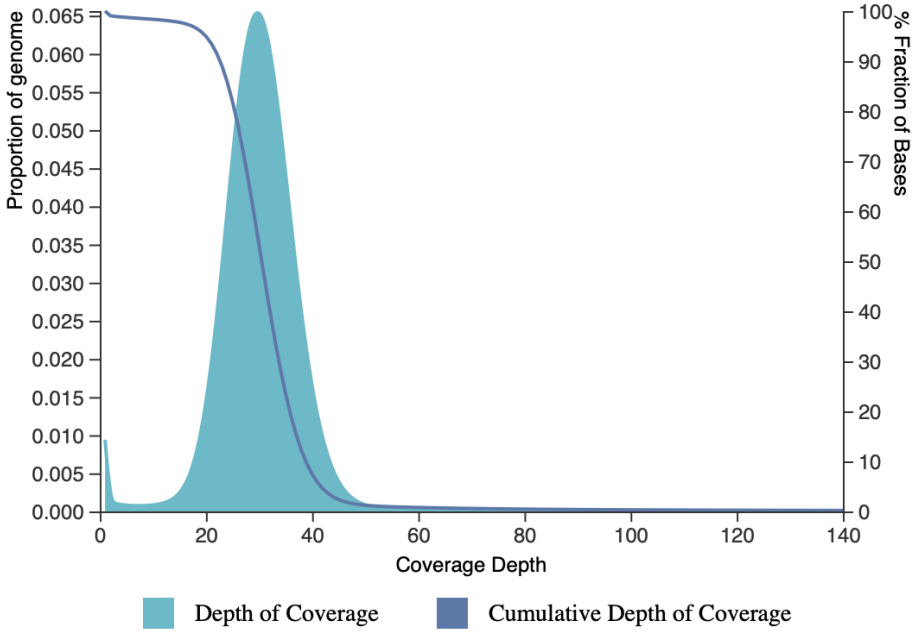

Figure 4.14: Depth of coverage. The figure is an example of histogram showing the depth of coverage for each sample.

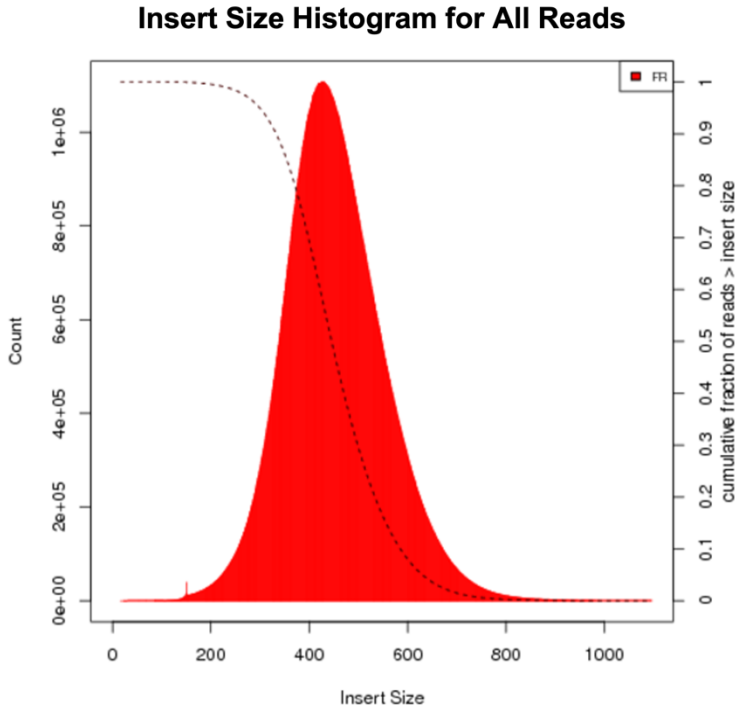

Figure 4.15: Insert Size of all Reads. Example diagram showing insert size of all reads (clustered data). The peak of the Gaussian corresponds to the average size (about 420 bp) of all fragments analyzed in each read of the sequence.

Sample ID	Fragment Length Median	Standard deviation
MP4	437	106,0
MP12	416	95,9
MP16	400	90,4
MP18	434	101,5
MP21	423	97,2
MP23	420	98,2
MP25	425	99,2
MP29	430	100,9
MP32	423	97,9
MP33	414	94,9
MP35	411	94,6

Table 4.9: Fragment Size and SD. The table shows the average size of the fragments used in the sequencing and the corresponding standard deviation (SD), for each sample analyzed.

Sample	Total yield	Total reads	GC%	Q20%	Q30%
MP4	117,195,722,714	776,130,614	42.83	96.67	91.7
MP12	119,014,014,582	788,172,282	40.97	96.98	92.26
MP16	117,384,095,516	777,378,116	41.18	96.67	91.69
MP18	121,484,075,602	804,530,302	40.91	96.66	91.6
MP21	113,240,808,932	749,939,132	41.4	96.92	92.11
MP23	119,922,012,614	794,185,514	40.87	96.51	91.27
MP25	116,449,798,720	771,190,720	41.31	96.87	92.03
MP29	118,409,997,670	784,172,170	40.95	96.5	91.23
MP32	116,758,354,234	773,234,134	41.59	96.54	91.42
MP33	111,606,834,610	739,118,110	41.31	96.97	92.19
MP35	111,146,426,852	736,069,052	40.91	96.48	91.3

Table 4.10: Fastq Statistics. In the table are specified the Fastq statistics data (Total yield, Total reads, GC % content, Q20% and Q30%).

4.4.3 Variant statistics

All variants identified by the next generation sequencing process are grouped into a single statistical diagram, in which the three broad categories of variants (SNPs/INDELs, CNVs, and SVs) are shown, divided into their corresponding subcategories, and their numerical frequency is specified for each.

For each of the analyzed patients a diagram is reported, which can be consulted in the Appendix. The example diagram is represented below (**Figure 4.16**).

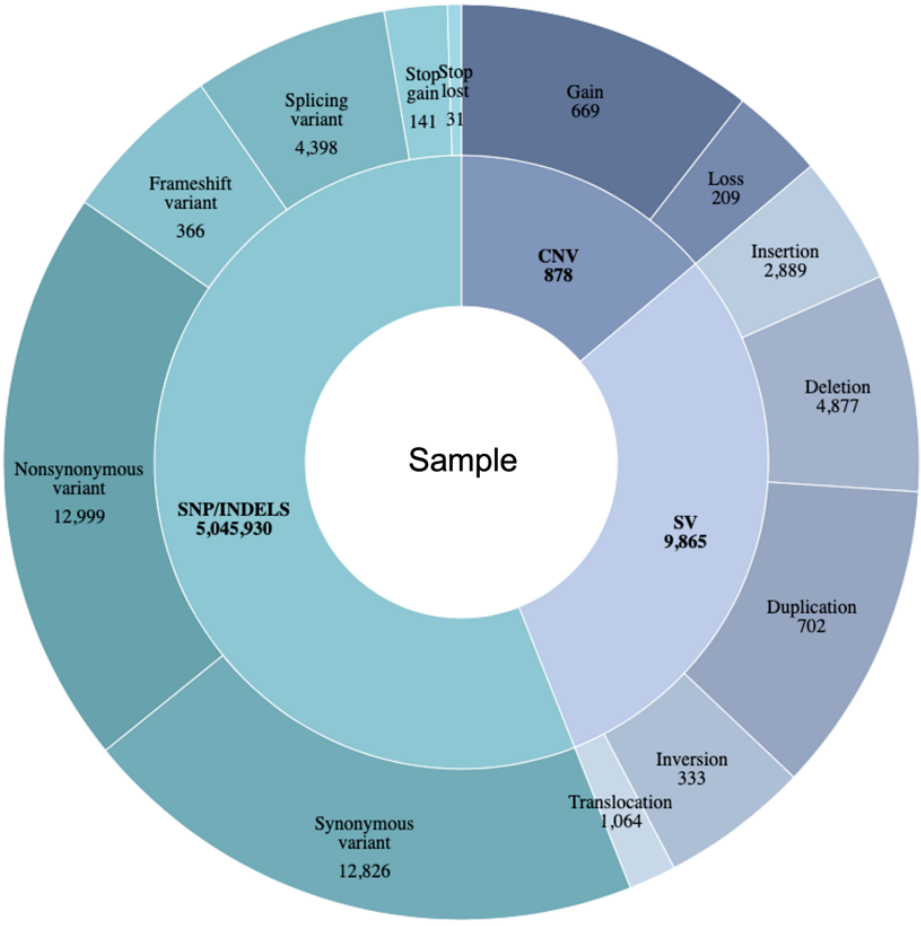

Figure 4.16: Variant Statistics Diagram. In the figure are reported in detail the number and the type of the possible genetic variations. The innermost circle shows the three major categories in three different colors (SNPs/INDELS, SVs and CNVs) and their subcategories, represented in the largest circle. Abbreviations: SNP: single nucleotide polymorphisms; INDELS: insertions or deletions; CNV: copy number variations; SV: structural variations.

Moreover, for each patient, an illustration of all the genetic, structural and copy number variations related to the structure of the human genome in its entirety, divided into autosomal and sex chromosomes, is represented. (**Figure 4.17)**.

Circos, an efficacious tool for displaying genetic structure variations and, generally, any other type of positional correlation across genomic regions, is a circular ideogram layout used to visualize relationships and associations between genomic positions and structural variant, copy number variations and SNPs and INDELs, extracted from whole genome sequencing data (Krzywinski *et al.*, 2009).

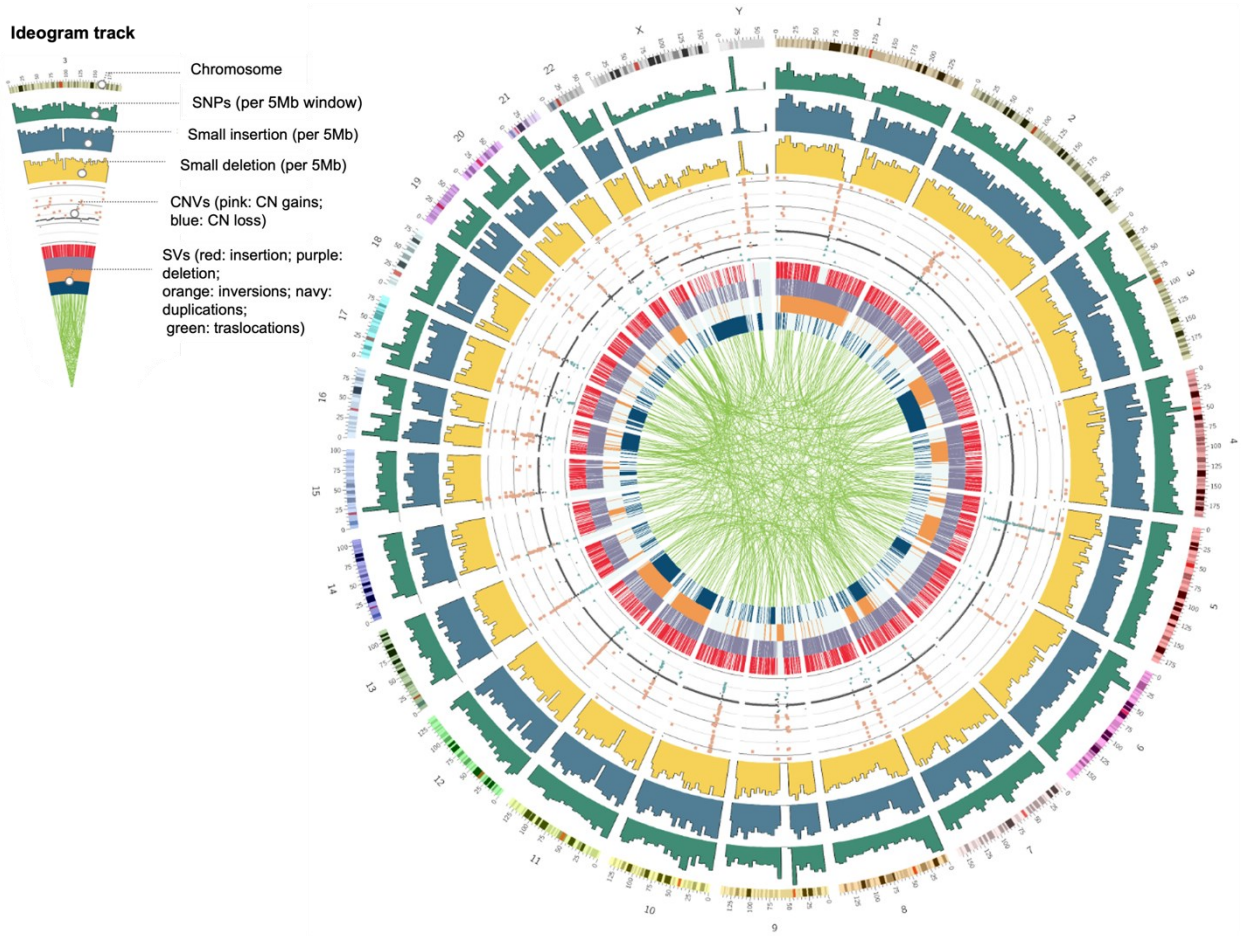

Figure 4.17: Circos ideogram. In the circular ideogram layout on the right, an example of results of all the 22 human autosomic chromosomes and the two sexual chromosomes (X and Y) and the variations found inside the genomic regions, for each sample analyzed is represented. On the left, the scheme to use as a key to read the diagram is specified. Abbreviations: SNPs: single nucleotide polymorphisms; CNVs: copy number variations; SVs: structural variations; Mb: mega bases.

4.4.3.1 Copy Number Variation (CNV)

A copy number variation (CNV) is a structural variation characterized by a stretch of DNA that varies, in copy number, from individual to individual, being duplicated in some subjects, and sometimes even tripled, quadrupled, and so forth. CNVs have long been associated with chromosomal rearrangements and specific genomic syndromes. Although it has long been recognized that some cancers are associated with high copy number of particular genes, the extent to which CNVs contribute to human disease is not yet known and research is still developing. Thus, it appears probable that, at least in humans, CNVs constitute a considerable amount of genetic variation. Because many CNVs include genes that drive differential levels of gene expression, CNVs may represent a significant portion of normal phenotypic variation (Freeman *et al.*, 2006; Zhang *et al.*, 2009).

In the samples analyzed in this study, the copy number variations (gains and losses) were on average just over 650 and about 200, respectively (**Table 4.11**).

Sample ID	Copy number gains	Copy number losses
MP4	630	163
MP12	669	209
MP16	640	183
MP18	667	259
MP21	623	190
MP23	716	214
MP25	737	218
MP29	754	219
MP32	561	184
MP33	572	173
MP35	660	181

Table 4.11: Copy Number Variations (CNVs). The table displays in detail the copy number variations (gains and losses) identified among the samples.

4.4.3.2 Structural Variation (SV)

Structural variation (SV) is commonly defined as a genomic region of DNA, approximately 1 kb and larger in size, that may include duplications, genomic imbalances (insertions and deletions), inversions and translocations. Some studies have established that about 5% to 13% of the human genome as being structurally diverse and rearranged in the normal population, affecting more than 800 independent genes that may influence on human phenotypic differences (Sharp, Cheng and Eichler, 2006; Sudmant *et al.*, 2015).

- Duplication: a region of DNA is duplicated and both copies end up in the same chromosome.
- Insertion: extra base pairs are inserted into the DNA sequence.
- Deletion: a region of DNA is lost or deleted.
- Inversion: a region of DNA is put in reverse.
- Translocation: two non-homologous chromosomes exchange DNA regions.

Among the samples analyzed in this study, the average rate of occurrence is about 680 duplications, about 3000 or slightly less for insertions and 5000 deletions, 1100 translocations, while, with regard to inversions, were found about 320, per subject (**Table 4.12**).

Sample ID	Duplications	Insertions	Deletions	Inversions	Translocations
MP4	715	2.788	5.072	302	1.084
MP12	702	2.889	4.877	333	1.064
MP16	721	2.867	4.909	336	1.194
MP18	686	2.858	4.997	312	1.060
MP21	700	3.132	5.672	329	1.146
MP23	657	2.967	5.114	326	1.074
MP25	679	2.924	5.023	319	1.168
MP29	719	2.861	4.940	316	1.124
MP32	662	2.918	4.971	342	1.162
MP33	668	2.769	4.861	319	1.070
MP35	637	2.680	4.796	329	1.112

Table 4.12: Structural Variations (SVs). In the table are reported in detail the number of the structural variations (duplications, insertions and deletions, inversions and translocations) identified among the analyzed samples.

4.4.3.1 SNP and INDEL

The rate of variants per individual is estimated to be about 4.5 million within the human genome, which is characterized by a length of about 3.1 billion base pairs and consists of about 20 thousand coding genes. However, it has been calculated that nearly 2 million variants have an allele frequency greater than 0.5, so there is actually one variant per 1200 bp.

In the samples analyzed in this study, the variants identified per subject were, on average, about a little more than 4 million. Of these, about 10% were classified as small deletions or small insertions, respectively (nearly 500,000); synonymous variants and missense variants were calculated to be, on average, just under 13,000 each, while the splice region variants turned out to be a little more than about 4 thousand; regarding the stop gained and stop loss, they are a little less than 150 and 35 for each subject, respectively; frameshift variants are about 340 on average, while, finally, inframe insertions and inframe deletion are about 200 or a little more for both of them (**Figure 4.18, Table 4.13**).

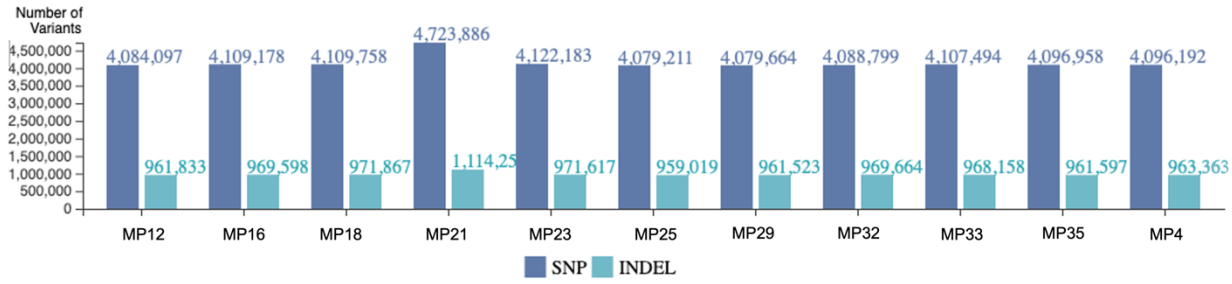

Figure 4.18: SNP and INDEL. The bar graphic illustrates in detail the number of SNPs and INDELs among the samples.

Sample ID	SNPs	Small insertions	Small deletions	Synonymous variant	Missense variant	Splice region variant	Stop gained	Stop loss	Frameshift	Inframe insertion	Inframe deletion
MP4	4.096.192	472.068	491.295	12.819	12.869	4.364	146	26	323	207	229
MP12	4.084.097	470.934	490.899	12.826	12.999	4.398	141	31	366	199	230
MP16	4.109.178	474.311	495.287	12.886	13.001	4.330	144	33	323	196	206
MP18	4.109.758	475.165	496.702	12.869	13.028	4.359	137	34	344	211	246
MP21	4.723.886	540.960	573.292	15.050	14.803	5.096	153	42	354	217	272
MP23	4.122.183	475.372	496.245	12.819	12.815	4.373	148	30	332	207	222
MP25	4.079.211	469.130	489.889	12.866	13.013	4.273	146	36	343	203	222
MP29	4.079.664	469.930	491.593	12.755	12.825	4.235	138	32	355	194	229
MP32	4.088.799	473.484	496.180	12.683	12.752	4.273	147	32	332	208	227
MP33	4.107.494	472.816	495.342	12.787	12.764	4.326	147	32	321	213	233
MP35	4.096.958	469.953	491.644	12.725	12.667	4.340	146	35	347	203	214

Table 4.13: Genetic variants and categories. Detail of the number of genetic variants, divided into categories, of each patient analyzed.

4.4.4 Copy Number Variations

Genomic analysis may produce several amounts of information, although there are some potential limitations to conducting genomic analysis with Next Generation Sequencing (NGS).

When genomic testing is done for the purpose of identifying underlying genetic causes of patients' symptoms and diseases, there may be results that are directly correlated to the manifestation of symptoms (called primary results or findings) but also results that, while having medical significance, are not related to the disease being tested for (usually called secondary or incidental results). Examples of secondary genetic findings may include susceptibility to developing future diseases, carrier status of a gene involved in some disease (while not showing the condition), and pharmacogenomic findings, related to differences in how a person can process medications or susceptibility to developing adverse reactions.

The first assessment, in order of priority, that we conducted on the whole genome sequencing results concerned structural variants, and in particular the copy number variations.

Of all the copy number variations (gains and losses) analyzed in the patient cohort, which averaged just over 650 and about 200 for each sample, respectively, regions in sharing among all patients analyzed and among the various subgroups were first extracted. Of these, only a small number of CNVs (7 regions in total) were found to be present in all NMS patients.

Moreover, among the category of NMS patients who took at least one FGA as a medication 15 CNVs, of which 8 were peculiar to these category, were found to be shared among the 6 patients; 12 CNVs, of which 5 were exclusive to this group, were found to be shared among patients who took at least one SGA as a medication (n=7); 10 CNVs were shared by G-carrier subjects (n=7), of which only 3 were found to be specific to these patients; finally, 28 CNVs, of which 21 were peculiar to these subjects, were shared by GG-carrier patients (n=4) (**Table 4.14**).

CNVs among NMS patients				
ALL Patients (n=11)	**FGA Patients (n=6)**	**SGA Patients (n=7)**	**G- carrier Patients (n=7)**	**G-non carrier Patients (n=4)**
7 CNVs	15 CNVs (8 + 7)	12 CNVs (5 + 7)	10 CNVs (3 + 7)	28 CNVs (21 + 7)

Table 4.14: CNVs shared among all NMS patients. In the table are detailed the numbers of CNVs shared among all NMS patients and divided by patient categories based on drug medications (FGA or FGA) or polymorphism detected (G-carrier or G-non carrier).

Firstly, we searched and identified the frequencies of the 7 genetic regions, detected among all 11 NMS patients analyzed, through the consultation of the database "DECIPHER" (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) (https://www.deciphergenomics.org). The aim was to understand whether these CNVs were already described and represented in patients, or in association with pathologies, or could be a peculiar trait in common among our NMS patients.

DECIPHER is a web database that collects and documents data of genomic variations, chromosomal abnormalities and pathogenic sequence variants, derived from DNA analysis of patients, which are mapped to the human genome using other databases such as UCSC Genome Browser and Ensembl. In DECIPHER database are reported 43949 open access copy number variants, 11259 open access sequence variants and 175173 phenotype observations among 42678 patients.

The frequency of the 7 common CNVs detected among all 11 NMS patients, calculated as a percentage of the number of patients who had these variants reported in DECIPHER, among the 42678 subjects analyzed, ranged from 0% to 0.288% (**Table 4.15**).

CNVs AMONG NMS PATIENTS AND SUBJECTS IN DECIPHER								
Chrom	Start	End	Predicted copy number	Type of alteration	Subject in Decipher with CNVs	Subject in Decipher with loss or gain	N° subject in Decipher	% Frequency of CNVs
chr12	8.410.000	8.440.000	0	loss	74	32	42678	0,074980083
chr13	111.670.000	111.790.000	1	loss	163	123	42678	0,288204696
chr16	21.500.000	21.580.000	4	gain	68	22	42678	0,051548807
chr16	34.960.000	35.070.000	1	loss	13	2	42678	0,004686255
chr17	21.670.000	21.840.000	3	gain	44	19	42678	0,044519425
chr17	25.930.000	26.570.000	0	loss	8	3	42678	0,007029383
chr21	0	5.160.000	1	loss	7	0	42678	0

Table 4.15: CNVs Frequency. In the table are reported the 7 CNVs in common among NMS patients and their relative frequencies calculated from the data reported in DECIPHER.

However, the ranges of the copy number variations in DECIPHER that overlap with those we analyzed in our sample, are more extended, being on average about 4-6 Mb, while those reported in our sample ranged from 10 to 640 kb, with one exception on chr 21 (of 5.15 Mb). Such small variations, on the order of kb, are not common among patients analyzed in the database, and this finding allows us to assume with some accuracy that the variations we identified in our cohort of

NMS patients are not typically described in association with the development of some known pathology.

In order to determine whether these CNVs, found in share among all NMS patients and the various subgroups, were commonly frequent and therefore definable as a typical trait of the European population, or rather typical of these subjects, we proceeded with a bioinformatic analysis through Ensembl database (https://www.ensembl.org/) using data collected in 100 Genome project phase 3 and DGVa (Database of Genomic Variants archive, https://www.ebi.ac.uk/dgva/) for each identified genomic interval.

4.4.4.1 CNVs among all NMS patients

Regarding the 7 CNVs found shared among all patients who developed neuroleptic malignant syndrome as a result of antipsychotic medications intake, 5 were found to be losses whereas only 2 were detected as gains. These structural variants were mapped within 5 different chromosomes, and their length ranged from 30 kb minimum to a maximum length of 5.16 Mb.

Each genomic interval and copy number variations shared among all 11 NMS patients were analyzed in order to find CNVs and structural variations already described (**Table 4.16**). Of these, 2 CNVs, one loss within chromosome 12 and one gain within chromosome 16, were found to be very common in both the general population (all) and the European population, with a frequency of 100% and about 88% respectively (**Figure 4.19**).

CNVs among all NMS patients								
Chrom	**Start**	**End**	**Predicted copy number**	**Type of alteration**	**Length**	**Gene – Genomic location**	**CNVs**	**CNVs Frequence**
chr12	8.410.000	8.440.000	0	loss	30 kb	OR7E140P (unprocessed pseudogene), 1 novel transcript	esv3628468 (loss)	ALL: 96,4% (2414)- EUR: 100% (503)
							esv3628469 (loss)	ALL: 97,5% (2442) - EUR: 100% (503)
							esv3628470 (gain/loss)	ALL: 97,8% (2451) - EUR: 100% (503)
chr13	111.670.000	111.790.000	1	loss	120 kb	Chr13q34 - No genes	No structural data	-
chr16	21.500.000	21.580.000	4	gain	80 kb	MIR3680-1, SMG1P3 (pseudogene), SLC7A5P2 (transcribed processed pseudogene), 2 lncRNA, 1 unprocessed pseudogene	esv3638168 (gain)	ALL: 85,4% (2436) - EUR: 88,07% (497)
							esv3638173 (gain/loss)	(gain) ALL: 84,8 % (2436) - EUR: 87,7 (497)
							esv3638178 (gain)	ALL:85,7 (2435) - EUR: 88,5 % (495)
							esv3638182 (deletion)	(gain) ALL: 0% - not reported
chr16	34.960.000	35.070.000	1	loss	110 kb	CCNYL3 (unprocessed pseudogene), novel pseudogene, 3 LncRNA,	esv3638489 (deletion)	ALL: 0,18 % (9) - EUR: 0% - not reported
							esv3638491 (deletion)	ALL: 0,20 % (10) - EUR: 0.1% (1)
							esv3638492 (deletion)	ALL: 0,08 % (4) - EUR: 0% - not reported
							esv3638493 (deletion)	ALL: 0,02 % (1) - EUR: 0% - not reported
chr17	21.670.000	21.840.000	3	gain	170 kb	KCNJ18, 2 unprocessed pseudogenes	No structural data	-
chr17	25.930.000	26.570.000	0	loss	640 kb	Chr17- Centromeric region	No structural data	-
chr21	0	5.160.000	1	loss	5,16 Mb	Chr21p13 - No genes	No structural data	-

Table 4.16: CNVs shared among all NMS patients. In the table are listed the 7 CNVs that were shared among all the 11 NMS patients. Chromosome, genomic coordinates, predicted copy number variation, type of alteration as gain or loss, length of genomic regions, genes involved, CNVs ID and frequencies are listed. Abbreviations: kb, kilobases.

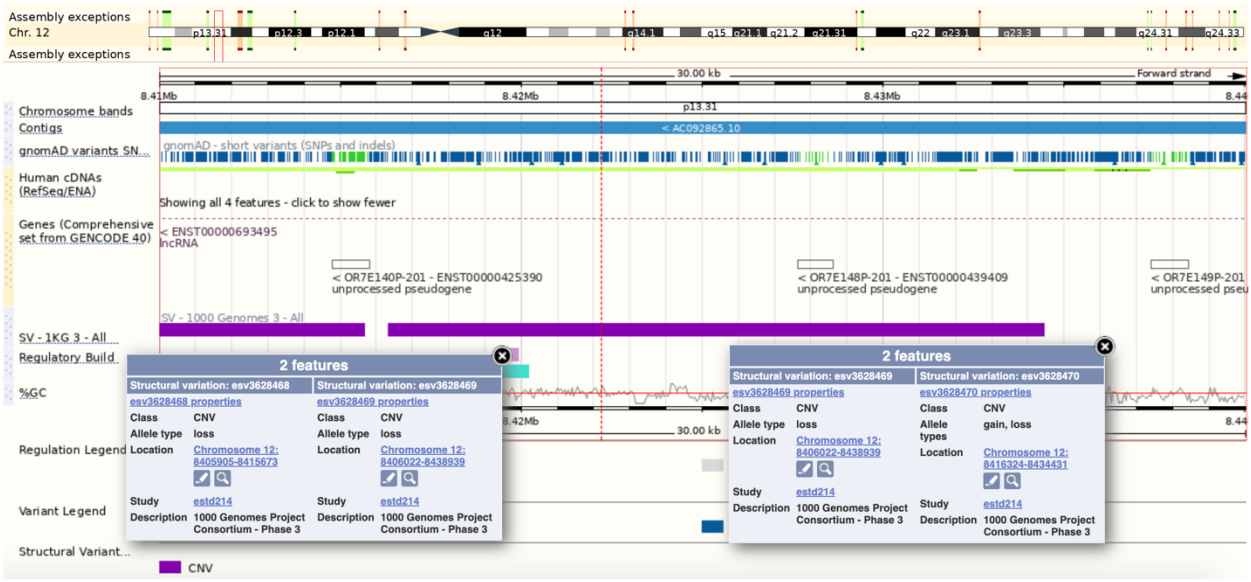

Figure 4.19: Genomic interval of chr12. The figure shows the detail of the 30 kb loss located on chr 12 and the 3 CNVs found within this region with 100% frequency among European population (https://www.ensembl.org/index.html).

The other 5 CNVs shared among all NMS patients were not found or reported in the databases for the European population. Thus, these CNVs appear to be unique and peculiar to these patients.

In three of these, a loss of 120 kb within Chr13q34, a loss concerning the first 5 Mb of chromosome 21, and another loss of 640 kb in the centromeric region of chromosome 17, respectively, no genes were mapped and thus no structural variants were identified.

A 110-kb CNV loss concerning chromosome 16, within which 3 lncRNAs and an unprocessed pseudogene (CCNYL3) were mapped, 4 deletion CNVs were described with a very low frequency in the general population (0.02-0.20%) and none in the European population, with the exception of the variant esv3638491, found in only one ethnic European subject among the 2,500 analyzed.

Finally, the last gain on chromosome 17 involves a 170-kb genomic interval in which is mapped the *KCNJ18* gene that encodes for the internal potassium rectifying channel of subfamily J. Also, for this CNV, no structural variants have been identified or reported in databases.

4.4.4.2 CNVs among FGA patients

There are 15 CNVs shared among FGA patients, 7 of which were already reported among all patients, and 8 (detailed in the table below) shared among this category of subjects, mapped within 5 different chromosomes. Of these, the first CNV on chromosome 9 is very common, with a frequency of 94% within the European population, and is also shared among the G-carrier group. The other 7 CNVs, on the other hand, reported with very low or no frequency within the European population, are also shared among the GG-carrier patient category. These structural variants included 6 gains and 2 losses, and their length ranged from 10 kb minimum to a maximum length of 340 kb (**Table 4.17**).

CNVs among FGA patients								
Chrom	Start	End	Predicted copy number	Type of alteration	Length	Gene – Genomic location	CNVs	CNVs Frequence
chr9	62.880.000	62.930.000	3	gain	50 kb	LERFS (lncRNA), FGF7P8 and CNTNAP3 (unprocessed pseudogenes)	esv3620515 (gain/loss)	(Gain) ALL: 82,47% (2500) - EUR: 93,94% (503)
chr16	30.190.000	30.290.000	3	gain	100 kb	BOLA2B, NPIPB12, NPIPB13, SLX1A, SLX1A-SULT1A3, SMG1P5, SULT1A3	No structural data	-
chr16	33.030.000	33.040.000	6	gain	10 kb	Chr16p11.2 - No genes	No structural data	-
chr16	34.070.000	34.080.000	3	gain	10 kb	Chr16p11.2 - No genes	esv3638480 (gain/loss)	(gain) ALL: 0,2% (8) - EUR: 0% - not reported
chr16	36.090.000	36.130.000	5	gain	40 kb	Chr16 - Centromeric region	No structural data	-
chr17	25.080.000	25.380.000	0	loss	300 kb	Chr17 - Centromeric region	No structural data	-
chr21	44.100.000	44.250.000	1	loss	150 kb	DNMT3L, GATD3, ICOSLG, LINC01678, PWP2, TRAPPC10	esv3647118 (deletion)	ALL: 0,06% (3) - EUR: 0% - not reported
							esv3647120 (indel)	ALL: 0,08% (4) - EUR: 0,47% (1)
							esv3647119 (indel)	ALL: 0,08% (4) - EUR: 0% - not reported
							esv3647122 (deletion)	ALL: 25,64% (1121) - EUR: 25,55% (228)
							esv3647123 (indel)	ALL: 3,31% (160) - EUR: 3,38% (33)
							esv3647125 (duplication)	(loss) ALL : 0% - not reported
chrX	155.700.000	156.040.895	2	gain	340,895 kb	SPRY3, VAMP7, IL9R, DPH3P2 (processed pseudogene), TMLHE (partial-promoter region), AMD1P2 (processed pseudogene), WASH6P (partial), TRPC6P (processed pseudogene)	esv3815906 (deletion)	ALL: 0,42% (16) - EUR: 0,65% (5)
							esv3816896 (deletion)	ALL: 0,29% (11) - EUR: 0,39% (3)
							esv3817707 (mobile element ins)	ALL: 0,19% (7) - EUR: 0% - not reported
							esv3816381 (mobile element ins)	ALL: 5,06% (184) - EUR: 10,18% (74)
							esv3817212 (mobile element ins)	ALL: 0,03% (1) (Han Chinese In Bejing)
							esv3817650 (deletion)	ALL:0,24% (9) - EUR: 0,39%(3)
							esv3817638 (gain)	ALL: 0,03%(1) (Finnish European)
							esv3816454 (deletion)	ALL: 4,95% (176) - EUR: 0,13% (1)
							esv3816185 (deletion)	ALL: 0,16% (6) - EUR: 0,26% (2)

Table 4.17: CNVs among FGA patients. In the table are listed the 7 CNVs (out of 15 total) that were shared among subjects who took at least one FGA as medication. Chromosome, genomic coordinates, predicted copy number variation, type of alteration as gain or loss, length of genomic regions, genes involved, CNVs ID and frequencies are listed. Abbreviations: kb, kilobases.

Within chromosome 16, three out of 4 identified gains have been mapped to genomic regions in which structural data have not been reported yet. Of these, only the first interval (chr16: 30,190,000-30,290,000) contains genes that encode for proteins (*BOLA2B, NPIPB12, NPIPB13, SLX1A, SLX1A-SULT1A3, SMG1P5, SULT1A3*). The last gain located on Chr16p11.2 involves a very rare variant, esv3638480, found in a total of 8 subjects (0.2%) of whom none were of European ethnicity.

Inside the chromosome 17, instead, has been found a loss of 300 kb in the centromeric region, for which no structural data have been found nor reported (**Figure 4.20**).

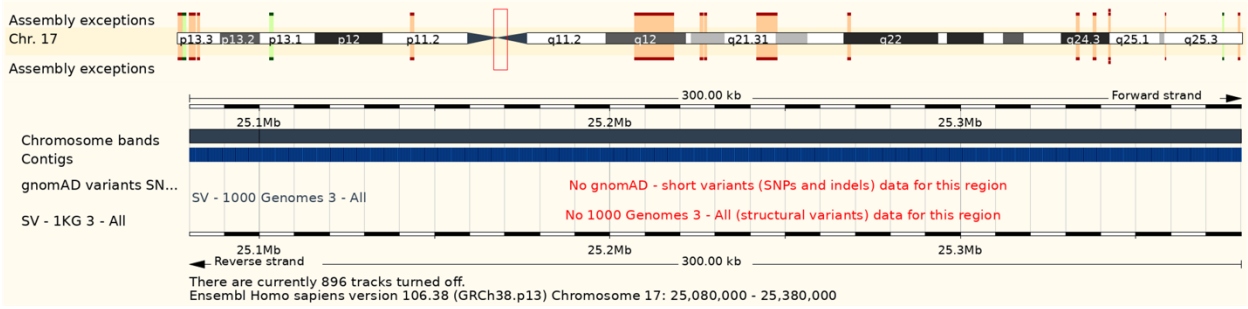

Figure 4.20: Genomic interval on chr17. In the figure is reported in detail the genomic location of the 300 kb loss in the centromeric region of chromosome 17. No structural variants or genetic elements are reported in this region (https://www.ensembl.org/index.html).

A loss of 150 kb was detected within the terminal region of chromosome 21, where several genes are mapped (*DNMT3L, GATD3, ICOSLG, LINC01678, PWP2, TRAPPC10*). In this interval, 6 CNVs have been discovered: 2 deletions (esv3647118 and esv3647122), the first one not detected and the second reported with a frequence of 25,5% in the European population; 3 indels (esv3647120, esv3647119, esv3647123) reported with very low or zero frequencies both in the global population and in the European one; the last CNV instead, esv3647125, is a duplication, therefore not present within the loss on chromosome 21.

The last CNV reported for the FGA patients group is a gain of more than 340 kb, located on the X chromosome. In this interval, several genes (*SPRY3, VAMP7, IL9R*) and processed pseudogenes are reported, and 9 structural variants have been described. Of these, however, 5 are described only as deletions thus no gains are repoted. Of the remaining 4, a gain (esv3817638) has been found in only one subject of European Finnish ethnicity, while the last 3 (described as "mobile element insertion"), the most frequent was found in the 10% of the European population (esv3816381), one (esv3817707) has not been reported among the European group and the last one (esv3817212) has been found in only one subject Han Chinese In Bejing (**Figure 4.21**).

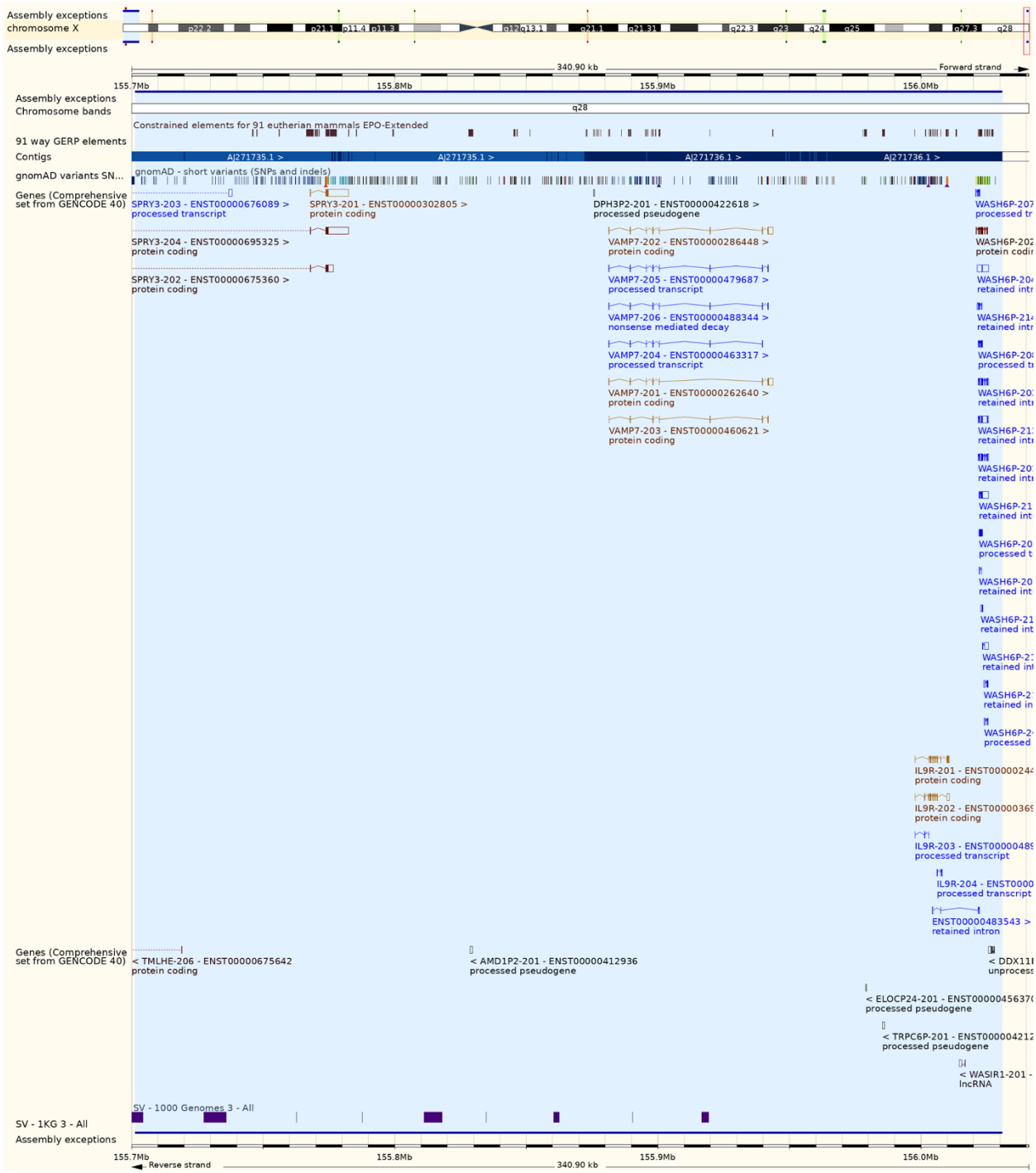

Figure 4.21: Genomic interval of chrX. In the figure is detailed the 340 kb gain located on chr X, the genes and the 9 CNVs found within this region (purple boxes) (https://www.ensembl.org/index.html).

4.4.4.3 CNVs among SGA patients

Among the group of 7 patients who took at least one second-generation antipsychotic and developed NMS, 5 CNVs were observed. These structural variants, 4 gains and 1 loss, were mapped within 4 different chromosomes, and their length ranged from 20 kb minimum to a maximum length of 60 kb.

Of these, only 1 CNV on the centromeric region of chromosome 3, for which no structural data are reported, was found to be exclusive to this group; 2 CNVs on chromosome 13 were also found to be shared among GG-carrier patients; the last 2 gains, on chr 16 and 21, respectively, were found to be shared also among G-carrier patients (**Table 4.18**).

CNVs among SGA patients								
Chrom	**Start**	**End**	**Predicted copy number**	**Type of alteration**	**Length**	**Gene – Genomic location**	**CNVs**	**CNVs Frequence**
chr3	90.510.000	90.550.000	4	gain	40 kb	Chr3 - Centromeric region	No structural data	-
chr13	16.170.000	16.230.000	0	loss	60 kb	Chr13 - Centromeric region	No structural data	-
chr13	63.060.000	63.080.000	5	gain	20 kb	2 lncRNA (novel transcript)	No structural data	-
chr16	34.160.000	34.180.000	5	gain	20 kb	LINC00273 (lncRNA), RNA5-8SP2 (RNA pseudogene)	esv3638483 (gain/loss)	(Gain) ALL: 0,18% (8) - EUR: 0%
chr20	28.910.000	28.960.000	3	gain	50 kb	Chr20 - Centromeric region	No structural data	

Table 4.18: CNVs among SGA patients. In the table are listed the 5 CNVs (out of 12 total) that were shared among subjects who took at least one SGA as medication. Chromosome, genomic coordinates, predicted copy number variation, type of alteration as gain or loss, length of genomic regions, genes involved, CNVs ID and frequencies are listed. Abbreviations: kb, kilobases.

For the three CNVs, one loss and two gains, detected in the centromeric region of as many chromosomes (chr3, chr13 and chr20) no structural data and frequencies were found for all of them yet. Likewise, no structural variants have been found either for the second region located on chromosome 13, where two lncRNAs have been mapped (**Figure 4.19**).

Figure 4.19: Genomic interval on chr13. Genomic location of the 20 kb gain in the chr13q21.31 region is detailde. Two lncRNA but no structural variants are reported (https://www.ensembl.org/index.html).

The last gain of 20 kb, identified among the group of SGA-patients, and shared also with G-carrier patients, is located near the centromeric region of the chr 16, precisely Chr16p11.2 (**Figure 4.20**). This region is only partially occupied by a lncRNA (*LINC00273*, long intergenic non-protein coding RNA 273) and a RNA-pseudogene RNA5-8SP2 (RNA, 5.8S ribosomal pseudogene 2). The esv3638483 variant, located at the beginning of the region and partially overlapping with *LINC00273*, has been described as both a loss and a gain, although the gain is less frequent than the loss, indeed it is reported in 8 subjects (0.18%) among the general population and not detected among European subjects.

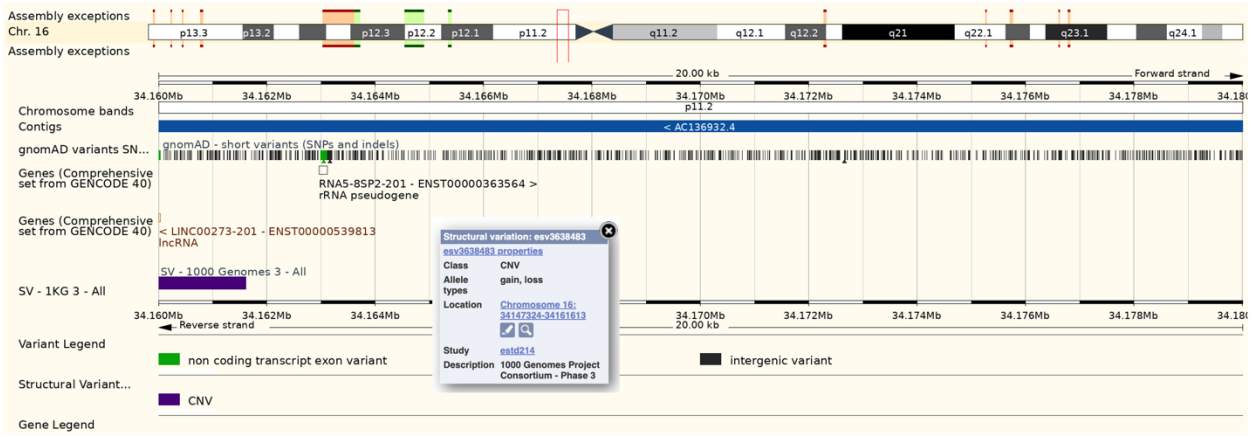

Figure 4.20: Genomic interval on chr16. In the figure is detailed the genomic location of the 20 kb gain in the Chr16p11.2. One lncRNA overlapping the esv3638483 variant and one rRNA pseudogene are reported in this region. The purple box represents the CNV detected (https://www.ensembl.org/index.html).

4.4.4.4 CNVs among G-carrier patients

Among the group of 7 NMS patients carrying at least one G allele of the rs1799732 genetic variant, 10 CNVs of which 7 were shared among all NMS subjects, and 3 were specific to this category, were found. These three structural variants, all of which gains, were mapped within 3 different chromosomes, and their length ranged from 20 kb minimum to a maximum length of 50 kb.

Of these, 1 CNV on chromosome 9, very common within the European population (f=94%) was also shared with FGA patients' group, while the other two were shared with SGA patient group (**Table 4.19**).

CNVs among G-carrier patients								
Chrom	Start	End	Predicted copy number	Type of alteration	Length	Gene – Genomic location	CNVs	CNVs Frequence
chr9	62.880.000	62.930.000	3	gain	50 kb	FGF7P8 (unprocessed pseudogene), LERFS (lncRNA), CNTNAP3 (unprocessed pseudogene)	esv3620515 (gain/loss)	(Gain) ALL: 82,47% (2500) - EUR: 93,94% (503)
chr16	34.160.000	34.180.000	5	gain	20 kb	LINC00273 (lncRNA), rRNA pseudogene	esv3638483 (gain/loss)	ALL: 0,18% (8) - EUR: 0% (not reported)
chr20	28.910.000	28.960.000	3	gain	50 kb	Chr20 - Centromeric region	No structural data	-

Table 4.19: CNVs among G-carrier patients. In the table are listed the 3 CNVs (out of 10 total) that were shared among G-carrier patients. Chromosome, genomic coordinates, predicted copy number variation, type of alteration as gain or loss, length of genomic regions, genes involved, CNVs ID and frequencies are listed. Abbreviations: kb, kilobases.

The second CNV reported for the G-carrier patients group, a gain of 20 kb located on the 16 chromosome (chr16p11.2) is shared also among the SGA patients group and previously reported (**Figure 4.20**).

The third and last 50 kb gain reported for the G-carrier patients group and shared also among the SGA patients group, is located in the centromeric region of Chr20, indeed no structural data or genomic elemets are reported within this interval (**Figure 4.21**).

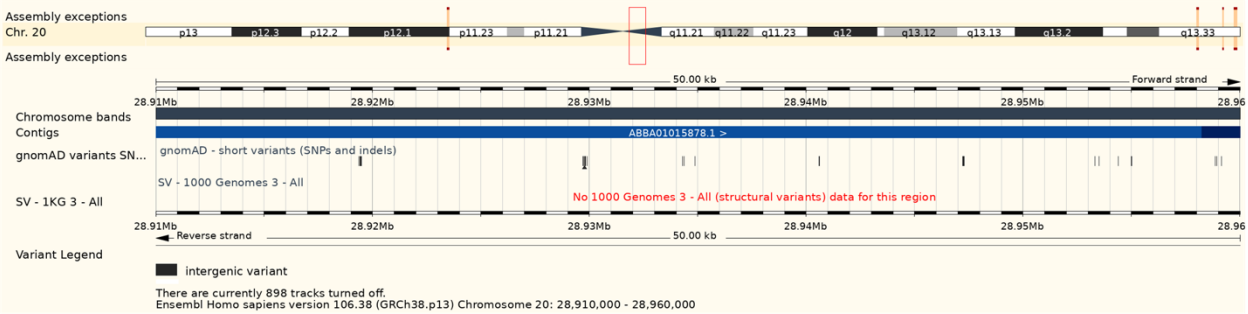

Figure 4.21: Genomic interval on chr20. In the figure is detailed the genomic location of the 50 kb gain in the centromeric region of chr20. No structural variants or genetic elements are reported in this region (https://www.ensembl.org/index.html).

4.4.4.5 CNVs among GG-carrier patients

In the last group analyzed, consisting of 4 NMS patients carrying no G allele of the genetic variant rs1799732, and defined as GG-carrier, 28 CNVs were reported, including 7 already described as shared among all NMS patients.

Of the 21 CNVs, 7 were also shared among FGA patients and 2 with the SGA patient group and respectively already described, whereas 12 were found to be exclusive to this group (**Table 4.20-4.21**).

These structural variants, 11 gains and 10 losses, were mapped within 11 different chromosomes, and their length ranged from 10 kb minimum to a maximum length of 460 kb.

CNVs among G-non carrier patients (a)								
Chrom	**Start**	**End**	**Predicted copy number**	**Type of alteration**	**Length**	**Gene – Genomic location**	**CNVs**	**CNVs Frequence**
chr1	121.780.000	122.240.000	1	loss	460 kb	Chr1 - Centromeric region	No structural data	-
chr5	47.930.000	47.950.000	1	loss	20 kb	Chr5 - Centromeric region	No structural data	-
chr8	39.370.000	39.530.000	1	loss	160 kb	ADAM5 (pseudogene), ADAM3A (pseudogene)	esv3616947 (deletion)	ALL: 26,6% (1095) - EUR: 42,8% (330)
							esv3616948 (deletion)	ALL: 26,6% (1095) - EUR: 42,84% (330)
							esv3616949 (deletion)	ALL: 26,68% (1097) - EUR: 42,94% (331)
							esv3616950 (deletion)	ALL: 26,66% (1097) - EUR: 42,9% (331)
							esv3616951 (deletion)	ALL: 26,6% (1096) - EUR: 42,84% (330)
chr10	42.150.000	42.170.000	5	gain	20 kb	Chr10q 11.21 - No genes	esv3622996 (duplication)	ALL: 0,12% (6) - EUR: 0% - not detected
chr11	1.010.000	1.020.000	3	gain	10 kb	AP2A2, MUC6	esv3625083 (duplication)	ALL: 0,02% (1) - EUR: 0% -not detected
							esv3625085 (duplication)	ALL: 0,06% (3) - EUR: 0,1% (1)
							esv3625094 (gain/loss)	(Gain) ALL: 0.04% (2) - EUR: 0.1% (1)
							esv3625096 (deletion)	(Gain) not detected
chr13	16.170.000	16.230.000	0	loss	60 kb	Chr13 - Centromeric region	No structural data	-
chr13	63.060.000	63.080.000	5	gain	20 kb	Chr13q21.31 - No genes	No structural data	-
chr16	30.190.000	30.290.000	3	gain	100 kb	BOLA2B, NPIPB12, NPIPB13, SLX1A, SLX1A-SULT1A3, SMG1P5, SULT1A3	No structural data	-
chr16	33.030.000	33.040.000	6	gain	10 kb	Chr16p11.2 - No genes	No structural data	-
chr16	34.070.000	34.080.000	3	gain	10 kb	Chr16p11.2 - No genes	esv3638480 (gain/loss)	(Gain) ALL: 0,2% (8) - EUR: 0% - not detected
chr16	34.080.000	34.100.000	5	gain	20 kb	Chr16p11.2- No genes	esv3638480 (gain/loss)	(Gain) ALL: 0,2% (8) - EUR: 0% - not detected
chr16	36.090.000	36.130.000	5	gain	40 kB	Chr16 - Centromeric region	No structural data	-

Table 4.20: CNVs among G-non carrier patients. In the table are listed the first 12 CNVs (out of 28 total) that were shared among G-non carrier patients. Chromosome, genomic coordinates, predicted copy number variation, type of alteration as gain or loss, length of genomic regions, genes involved, CNVs ID and frequencies are listed. Abbreviations: kb, kilobases.

CNVs among G-non carrier patients (b)								
Chrom	**Start**	**End**	**Predicted copy number**	**Type of alteration**	**Length**	**Gene – Genomic location**	**CNVs**	**CNVs Frequence**
chr17	21.290.000	21.350.000	3	gain	60 kb	MAP2K3	esv3640231 (duplication)	ALL: 0,04% (2) - EUR: 0,2% (2)
chr17	22.120.000	22.160.000	6	gain	40 kB	UBBP4 (pseudogene)	esv3640244 (mobile element ins)	ALL: 5,87% (249) - EUR: 9,54% (96)
chr17	24.430.000	24.740.000	0	loss	310 KB	Chr17 - Centromeric region	No structural data	-
chr17	25.080.000	25.380.000	0	loss	300 KB	Chr17 - Centromeric region	No structural data	-
chr17	25.790.000	25.920.000	0	loss	130 kb	Chr17 - Centromeric region	No structural data	-
chr20	1.580.000	1.610.000	0	loss	30 kb	SIRPB1 (unprocessed pseudogene)	esv3644983 (deletion)	ALL:81,85 % (2409) - EUR: 79,03 (485)
chr20	29.130.000	29.210.000	0	loss	80 kb	Chr20 - Centromeric region	No structural data	-
chr21	44.100.000	44.250.000	1	loss	150 kb	DNMT3L, GATD3, ICOSLG, LINC01678, PWP2, TRAPPC10	esv3647118 (deletion)	ALL: 0,06% (3) - EUR: 0%
							esv3647120 (indel)	ALL: 0,08% (4) - EUR: 0,47% (1)
							esv3647119 (indel)	ALL: 0,08% (4) - EUR: 0%
							esv3647122 (deletion)	ALL:25,64% (1121) - EUR: 25,55% (228)
							esv3647123 (indel)	ALL: 3,31% (160) - EUR: 3,38% (33)
							esv3647125 (duplication)	(loss) ALL : 0%
chrX	155.700.000	156.040.895	2	gain	340,895 kb	SPRY3, VAMP7, IL9R, DPH3P2 (processed pseudogene), TMLHE (partial-promoter region), AMD1P2 (processed pseudogene), WASH6P (partial), TRPC6P (processed pseudogene)	esv3815906 (deletion)	ALL: 0,42% (16) - EUR: 0,65% (5)
							esv3816896 (deletion)	ALL: 0,29% (11) - EUR: 0,39% (3)
							esv3817707 (mobile element ins)	ALL: 0,19% (7) - EUR: 0%
							esv3816381 (mobile element ins)	ALL: 5,06% (184) - EUR: 10,18% (74)
							esv3817212 (mobile element ins)	ALL: 0,03% (1) (Han Chinese In Bejing)
							esv3817650 (deletion)	ALL:0,24% (9) - EUR: 0,39%(3)
							esv3817638 (gain)	ALL: 0,03%(1) (Finnish European)
							esv3816454 (deletion)	ALL: 4,95% (176) - EUR: 0,13% (1)
							esv3816185 (deletion)	ALL: 0,16% (6) - EUR: 0,26% (2)

Table 4.21: CNVs among G-non carrier patients. In the table are listed the last 9 CNVs (out of 28 total) from chromosome 17, that were shared among G-non carrier patients. Chromosome, genomic coordinates, predicted copy number variation, type of alteration as gain or loss, length of genomic regions, genes involved, CNVs ID and frequencies are listed. Abbreviations: kb, kilobases.

Of the 12 CNVs shared exclusively within the 4 patients belonging to the GG-carrier group, 5 intervals (located inside chromosomes 1, 5, 17, 17 and 20) were mapped within the centromeric regions, therefore no gene or structural variant was found in these locations. Furthermore, on chromosome Chr10q 11.21, a gain of 20 kb, in which no genes or genetic elements, but only one

duplication has been detected. This CNV duplication (esv3622996) has a very low frequency, indeed it is reported in 5 subjects (0,12%) among the general population and not detected among European subjects.

One loss of 160 kb within chromosome 8 was reported. In this interval, two pseudogenes (*ADAM5* and *ADAM3A*) and 5 structural variants spanning the region (esv3616947, esv3616948, esv3616949, esv3616950 and esv3616951) have been described. All 5 of these CNVs have been reported as deletions, with a frequency within the general population of 26.6% and in the European population of approximately 43% (**Figure 4.22**).

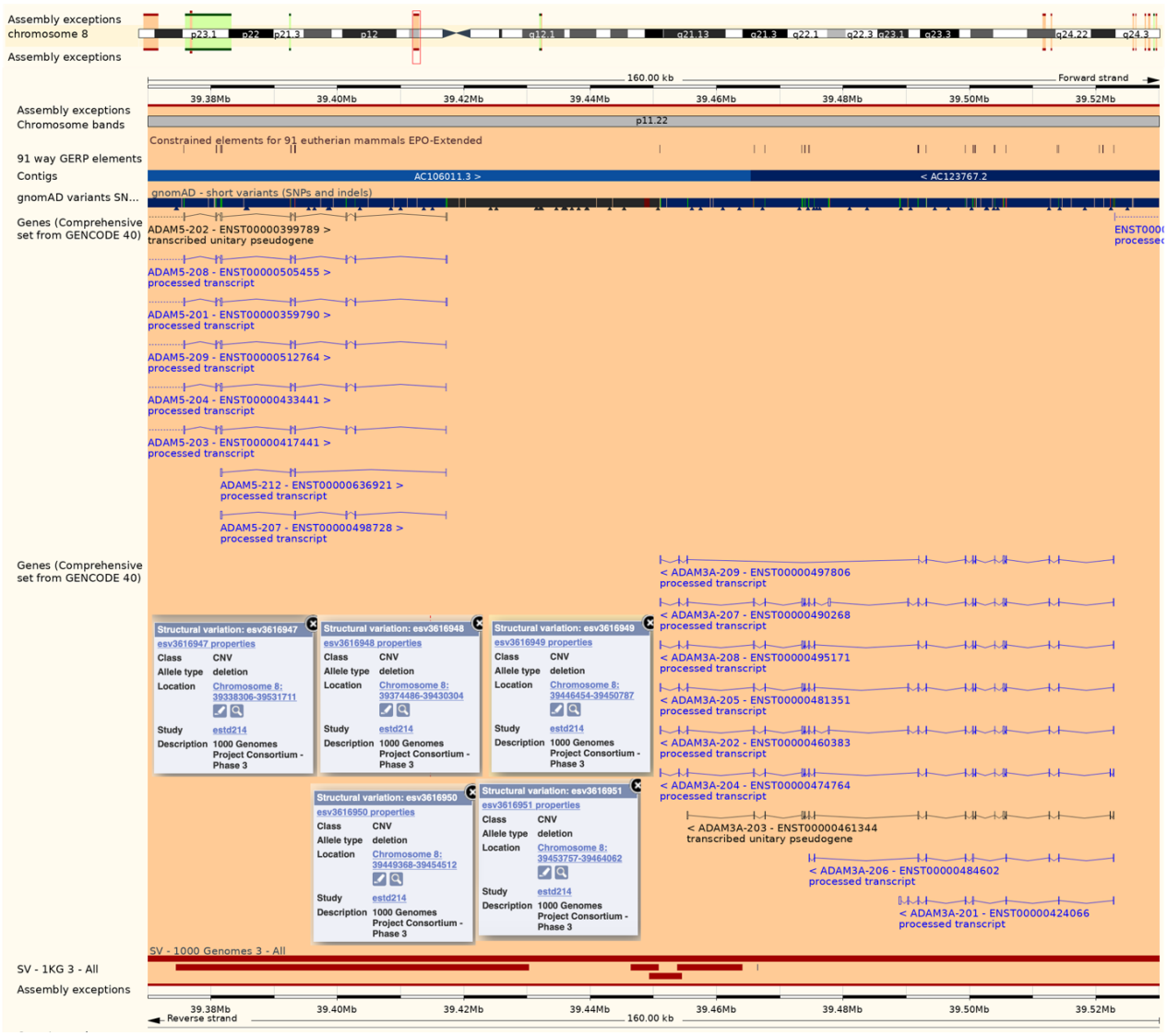

Figure 4.22: Genomic interval on chr8. In the figure is detailed the genomic location of the 160 kb gain in the Chr8. Two pseudogenes (*ADAM5 and ADAM3A*) overlapping the variants are reported in this region. The red boxes represent the CNV deletions detected (https://www.ensembl.org/index.html).

Within the 10 kb gain on chromosome 11, two genes (*AP2A2* and *MUC6*) were detected. Among these, four CNVs, two duplications (esv3625083 and esv3625085) and one gain (esv3625094) were found with very low or no frequency, respectively, while the last variant was described only as a deletion (esv3625096) (Figure 4.23).

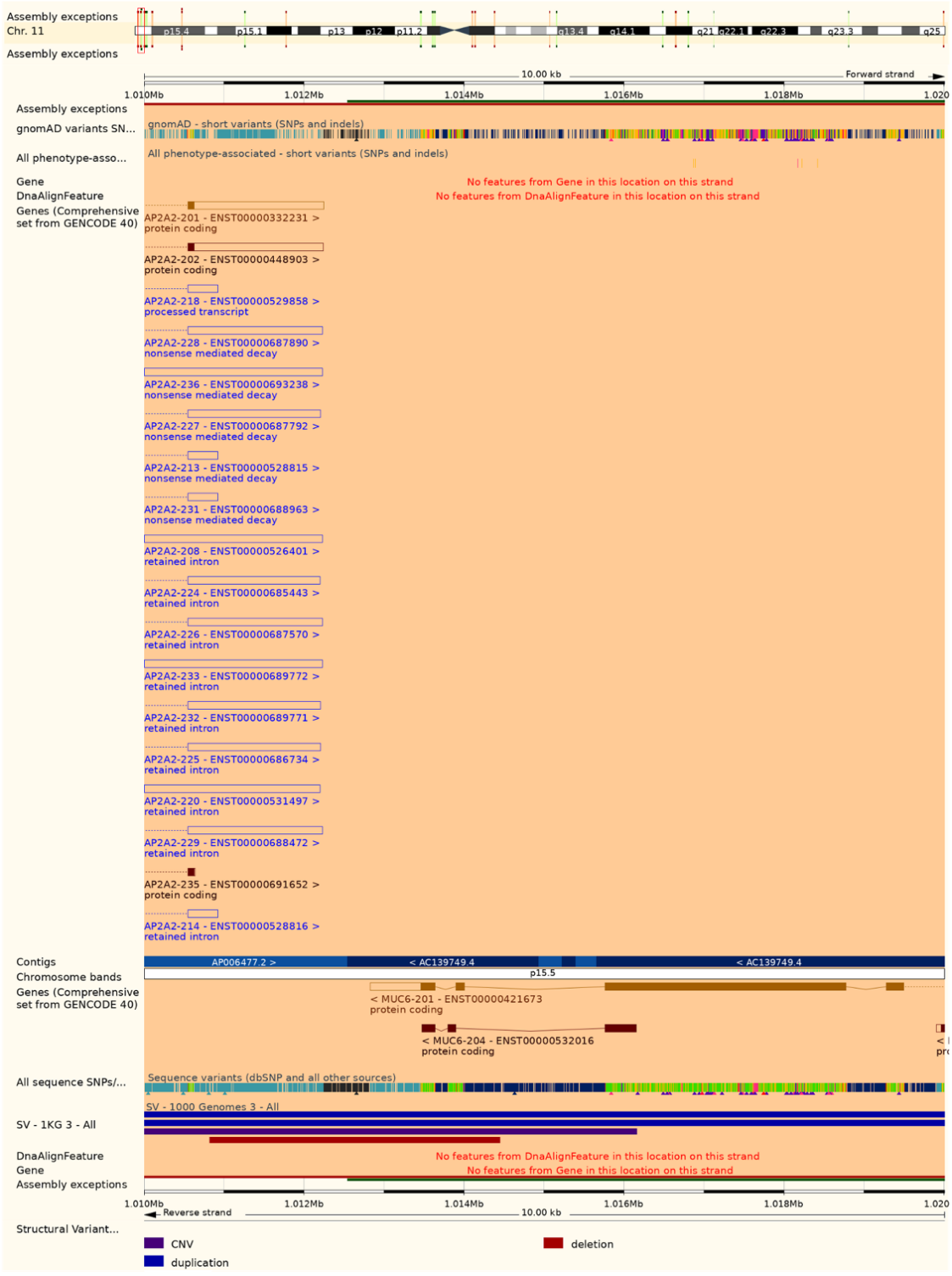

Figure 4.23: Genomic interval on chr11. In the figure is detailed the genomic location of the 10 kb gain in the Chr11. Two genes (*AP2A2 and MUC6*) overlapping the variants are reported in this region. The red boxes represent the CNV deletions detected, the blue boxes represent the gains/duplications (https://www.ensembl.org/index.html).

All the identified CNVs found within chromosome 16 were shared also among the FGA patient group (and therefore already described), except for a gain of 20 kb located precisely in position chr16p11.2 (genomic location chr16: 34,080,000-34,100,000). In this region, no genes or genomic elements have been reported; however, the structural variant esv3638480, described as both loss and gain, is present and very rare, as the gain was found in only 8 subjects in the general population (0,2%) and not detected in the European population.

Moreover, two gains of 60 kb and 40 kb, in two different positions on chromosome 17p11.2 were detected. In the first interval, one gene (*MAP2K3*) and one duplication (esv3640231) with very low frequency in general and European populations (0,04% and 0,2% respectively), were mapped, however, the duplication does not overlap with the gene region. In the second region, instead, located close to the beginning of the centromeric region of chromosome 17, a transcribed unprocessed pseudogene (*UBBP4*) and a small structural variant (esv3640244), described as a mobile element insertion of only 1 bp and detected with a frequency of 9.5% in the European population, that does not affect the pseudogene region, were mapped (**Figure 4.24**).

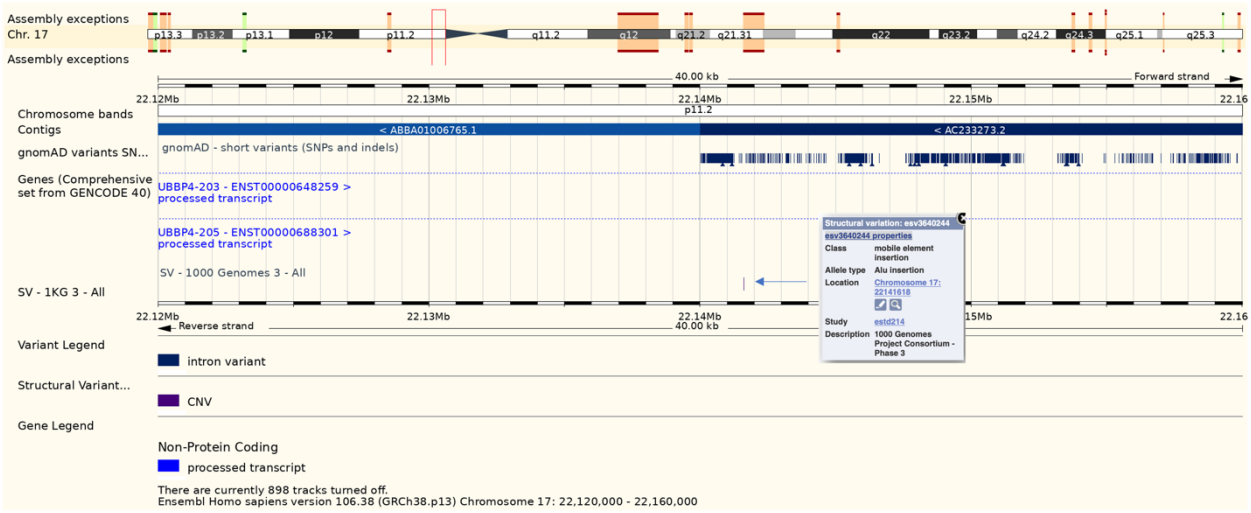

Figure 4.24: Genomic interval on chr17. In the figure is detailed the genomic location of the 40 kb gain in the Chr17. One pseudogene (*UBBP4*) overlapping the variants is reported in this region. The box displays the CNV gain detected, (https://www.ensembl.org/index.html).

The last detected CNV among the group of GG-bearing patients was characterized by a 30 kb loss located within chromosome 20. In this interval, a gene, a pseudogene and a large CNV, defined as deletion, have been mapped. This variant (esv3644983) spans the entire identified region and has been observed in approximately 82% of the world population and 79% of European subjects, indeed it is very frequent.

In conclusion, in-depth analysis of CNVs shows that many copy number variations are located in centromeric regions or chromosomal regions where no genes or structural variant have been mapped or reported yet (**Table 4.22**). It is noteworthy that most of the genes affected by CNVs, reported with very low or no frequencies, belong to both the G-noncarrier and FGA subgroups. In addition, shared CNVs were also found between the G-carrier and FGA subgroups.

Finally, regarding the 7 CNVs shared among all 11 NMS patients, only 5 (4 losses and 1 gain) were found to be uncommon among the European population, and among them, three lncRNA and only one gene affecting by a gain were identified. This gene, encoding for a potassium channel (*KCNJ18*) protein, would appear to be of interest regarding the manifestations of NMS (see Discussion and Conclusion below).

Genes, lncRNA and Pseudogenes					
	ALL patients (n=11)	**G-carrier (n=7)**	**G-non carrier (n=4)**	**FGA (n=6)**	**SGA (n=7)**
GENES	KCNJ18 (gain)	-	AP2A2, MUC6, MAP2K3	BOLA2B, NPIPB12, NPIPB13, SLX1A, SLX1A-SULT1A3, SMG1P5, SULT1A3	-
			BOLA2B, NPIPB12, NPIPB13, SLX1A, SLX1A-SULT1A3, SMG1P5, SULT1A3		
			DNMT3L, GATD3, ICOSLG, PWP2, TRAPPC10	DNMT3L, GATD3, ICOSLG, PWP2, TRAPPC10	
			SPRY3, VAMP7, IL9R	SPRY3, VAMP7, IL9R	
lncRNAs	3 lncRNA (loss)	LNC00273 (gain)	LINC01678, 2lncRNA (gain)	LINC01678	LINC00273 (gain), 2lncRNA (gain)
Pseudognes	CCNYL3 (loss), 2 unprocessed pseudogenes	rRNA pseudogenes (gain)	ADAM5, ADAM3A, UBBP4, SIRPB1 DPH3P2, AMD1P2, TRPC6P	DPH3P2, AMD1P2, TRPC6P	rRNA pseudogenes (gain)

Table 4.22: Genes, lncRNA and Pseudogenes shared among all patients. A summary of the CNVs and genes among NMS patient group and subgroups is detailed.

4.4.5 Structural Variants

The human genome is affected, on average, by approximately 5% of significant structural variants and rearrangements, such as deletions and insertions, inversions, translations, and tandem repeats. It has been estimated that these structural variants (SVs) affect approximately 800 genes and may influence more or less heavily the human phenotype.

Of all the structural variants analyzed in our cohort of patients, which averaged a little more than 10000, were first extracted the regions in sharing among all patients analyzed. The distribution of SVs was not homogeneous with respect to subcategories: deletions (about 5000 on average) were the most represented, followed by insertions (just under 2900), translocations (about 1100) and finally the least represented categories, duplications and inversions, about 700 and just over 300, respectively.

4.4.5.1 Structural variants among NMS patients

The structural variants, identified in shared among all 11 NMS patients and extrapolated through a bioinformatic analysis conducted with the use of BCFtools software, amounted to about 10% of the total variants found in each of the individuals, and precisely to 1024.

The list of these 1024 shared structural variants was analyzed on the Ensembl Variant Effect Predictor tool (VEP) in .vcf format. The SVs processed by the tool were in all 1006, of which 954 mapped on overlapped genes, 4027 mapped in overlapped transcripts and 2065 in overlapped regulatory features. Moreover, all of them were coding sequence structural variants (**Figure 4.25**).

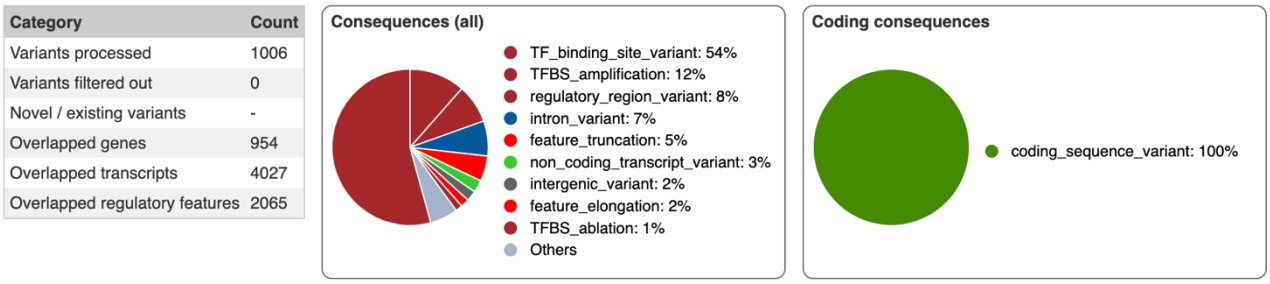

Figure 4.25: VEP results. A typical VEP results page is shown in the figure, containing a summary table and two pie charts, detailing statistics on genes, transcripts, regulatory features, all consequences and coding consequences (https://www.ensembl.org/info/docs/tools/vep/index.html).

Of the structural variants, we focused on those that could affect only the coding region, and thus have effects on protein assembly, structure, and function. Therefore, first we extracted the

variants that had "protein coding" as the Biotype filter, and "high" as the Impact. Of these, only two SVs, one on chr 14 and the other on chr X, were identified as a result of the filtering procedure.

On chromosome X the variant identified was a duplication that spans a total of almost 2.8 MB, in which 32 genes are involved (**Table 4.23**). In this region, a multitude of smaller and larger structural variants are already described in databases. However, the X chromosome, which has been extensively studied before, is a very fragile structure, in which some regions are duplicated by default.

On chromosome 14, instead, the variant identified was a smaller deletion of about 140 kb, in which only one gene (*MIPOL1*), was found (**Table 4.23**). Two SVs, already described and reported in databases, were found within the beginning of this region: esv3634083, a deletion of 4514 bp reported in only 1 subject of African ethnicity, and esv3634084 deletion of 2833 bp, with a frequency of 0,38% in all population and 0,6 in the European population according to 1000 Genomes Project Phase 3.

SVs among all NMS patients											
Chrom	**Start**	**End**	**Allele**	**Length**	**Consequence**	**Impact**	**Biotype**	**Gene name**			
14	37162401	37302022	deletion	140 kb	transcript_ablation	HIGH	Protein_coding	MIPOL1			
X	52857690	55652517	duplication	2,79 Mb	transcript_amplification	HIGH	Protein_coding	ALAS2	GPR173	MAGEH1	RIBC1
								APEX2	HSD17B10	MTRNR2L10	SMC1A
								FAM104B	HUWE1	PAGE2	TRO
								FAM120C	IQSEC2	PAGE2B	TSPYL2
								FAM156B	ITIH6	PAGE3	TSR2
								FGD1	KANTR	PAGE5	USP51
								FOXR2	KDM5C	PFKFB1	WNK3
								GNL3L	MAGED2	PHF8	XAGE3

Table 4.23: Protein-coding and high-impact SVs. In the table the 2 filtered SVs by protein-coding and high-impact biotype found to be shared among NMS patients are specified. Genomic location, Allele description, length and consequences are also detailed.

In the second instance we limited ourselves to filtering and extracting variants that had only "protein coding" as Biotype. Therefore, 435 structural variants were identified, divided into 311 deletions, 21 duplications, 84 insertions and 19 inversions. In these 435 intervals affected by as many SVs, 473 genes mapping within were identified.

Of these 473 genes, we attempted to identify shared pathways that might be somehow compromised by the presence of these 435 structural variants. Analysis was performed against Reactome version 80, a curated database of pathways and reactions in human biology (https://reactome.org). The result showed that 264 of the 473 sample identifier genes were found in Reactome, where 1021 pathways were affected by at least one of them, while 209 genes yielded

no results. This is an overrepresentation analysis, which is a statistical test (hypergeometric distribution) that determines whether certain Reactome pathways are overrepresented (enriched) in the submitted data. This test produces a probability score, which is corrected for the false discovery rate using the Benjamani-Hochberg method. Reactome pathways are organized in a hierarchy. A genome-wide overview of the results of pathway analysis is shown in the figure. (**Figure 4.26**).

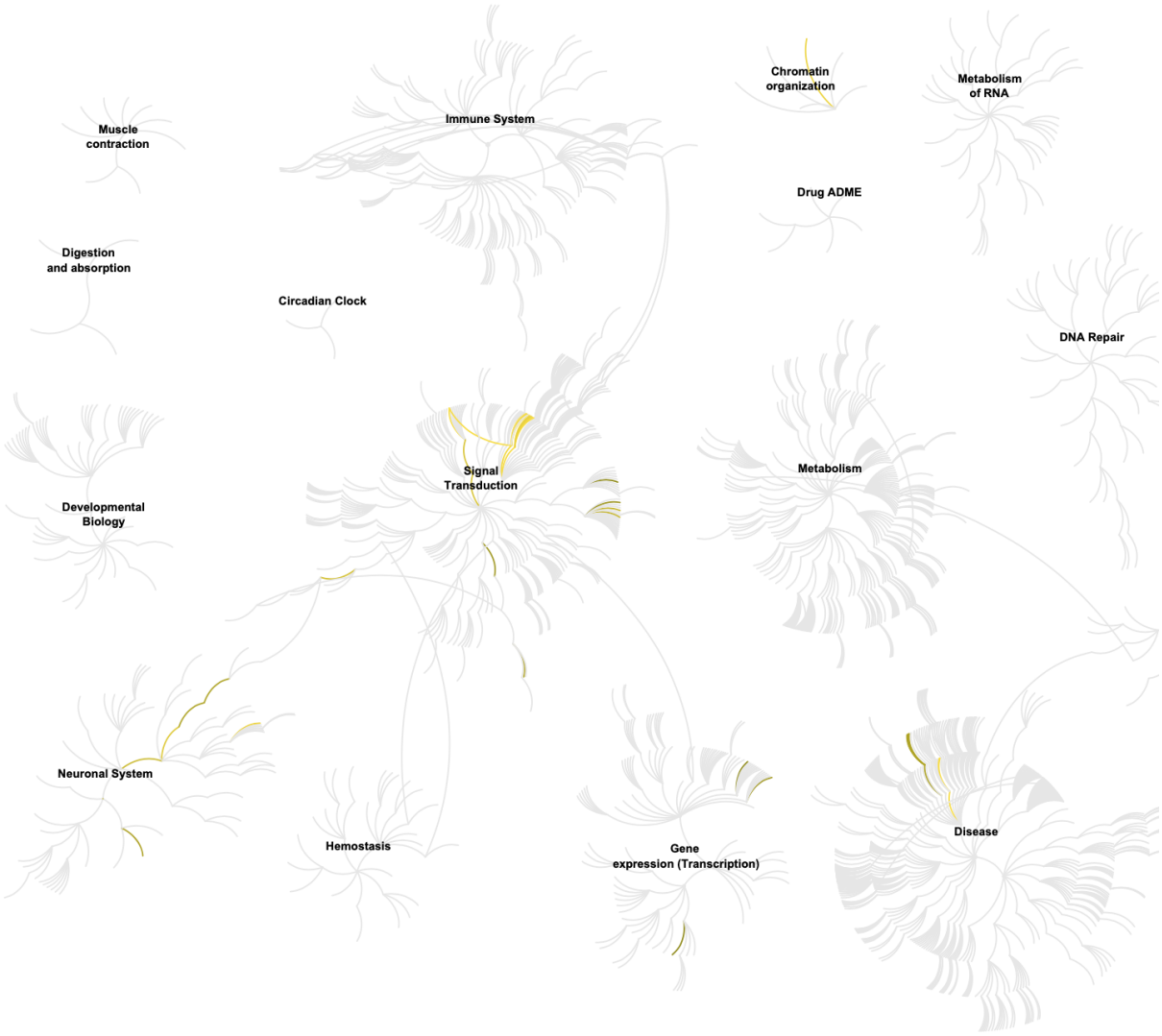

Figure 4.26: Genome-wide overview. In the figure a genome-wide overview of the pathways analysis is detailed. The center of each circular path is the root of one of the higher-level paths. Each step away from the center represents the next lowest level in the path hierarchy. Gray colour is for the non-significant over-represented path, yellow is for the most represented ones (https://reactome.org).

The most significant pathways sorted by p-value, represented in yellow line in the figure, are detailed in the table below (**Table 4.24, Figure 27**). Most of the 264 genes identified in the Reactome database are involved in disease (especially cancer), signal transduction, gene expression (transcription), chromatin organization, and the neuronal system pathways. Thus, significant structural changes in genes involved in these pathways, such as those identified as a result of next-generation sequencing analysis, may affect not only protein expression, but also have a cascading effect on the underlying biochemical pathways.

Pathway name	Entities found/total	Entities ratio	p-value	FDR	Reactions found/total	Reactions ratio
Signaling by ERBB2 TMD/JMD mutants	8/30	0.002	1.54E-5	1.19E-2	11/13	0.001
Signaling by ERBB2 KD Mutants	8/35	0.002	4.52E-5	1.19E-2	12/17	0.001
ERBB2 Activates PTK6 Signaling	6/18	0.001	5.35E-5	1.19E-2	2/2	0
Signaling by ERBB2 in Cancer	8/36	0.002	5.5E-5	1.19E-2	46/62	0.004
ERBB2 Regulates Cell Motility	6/19	0.001	7.19E-5	1.3E-2	2/2	0
GRB2 events in ERBB2 signaling	6/21	0.001	1.23E-4	1.68E-2	4/4	0
SHC1 events in ERBB4 signaling	6/21	0.001	1.23E-4	1.68E-2	4/4	0
SHC1 events in ERBB2 signaling	7/36	0.002	3.55E-4	3.83E-2	4/6	0
Downregulation of ERBB2 signaling	7/36	0.002	3.55E-4	3.83E-2	6/14	0.001
Signaling by ERBB4	10/82	0.005	8.44E-4	8.35E-2	52/52	0.004
PI3K events in ERBB2 signaling	5/22	0.001	1.25E-3	1.13E-1	4/7	0.001
PI3K events in ERBB4 signaling	4/15	0.001	2.18E-3	1.81E-1	2/2	0
Signaling by ERBB2	8/68	0.005	3.36E-3	2.59E-1	31/46	0.003
Long-term potentiation	5/31	0.002	5.38E-3	3.66E-1	3/17	0.001
HDMs demethylate histones	5/31	0.002	5.38E-3	3.66E-1	7/17	0.001
Nuclear signaling by ERBB4	6/47	0.003	7.22E-3	4.43E-1	34/34	0.002
Downregulation of ERBB4 signaling	3/11	0.001	7.39E-3	4.43E-1	5/5	0
GABA receptor activation	7/68	0.005	1.17E-2	6.68E-1	11/12	0.001
Signaling by Non-Receptor Tyrosine Kinases	7/71	0.005	1.45E-2	6.79E-1	4/53	0.004
Signaling by PTK6	7/71	0.005	1.45E-2	6.79E-1	4/53	0.004
G-protein mediated events	7/73	0.005	1.66E-2	6.79E-1	19/41	0.003
GRB7 events in ERBB2 signaling	2/6	0	1.97E-2	6.79E-1	1/1	0
Neurotransmitter receptors and postsynaptic signal transmission	15/232	0.015	2.07E-2	6.79E-1	34/109	0.008
RHOA GTPase cycle	11/154	0.01	2.36E-2	6.79E-1	3/6	0
RHOC GTPase cycle	7/79	0.005	2.42E-2	6.79E-1	1/6	0
Neuronal System	26/489	0.032	2.89E-2	6.79E-1	59/216	0.016

Table 4.24: Over-represented pathways. In the table the most significant pathways, sorted by p-value, are detailed. Abbreviations: FDR, false discovery rate.

It is certainly interesting to note that, among the top 26 pathways overrepresented on the basis of p-value, “Neurotransmitter receptors and postsynaptic signal transmission” and “Neuronal system” pathways are involved, with a total of 15 and 25 proteins detected respectively. Although these findings will certainly need to be corroborated by experimental and functional data, to determine how SVs affect the function of genes and proteins, what changes are the result and what

is the final effect on the signal transduction cascade, are still important results that confirm the data collected so far.

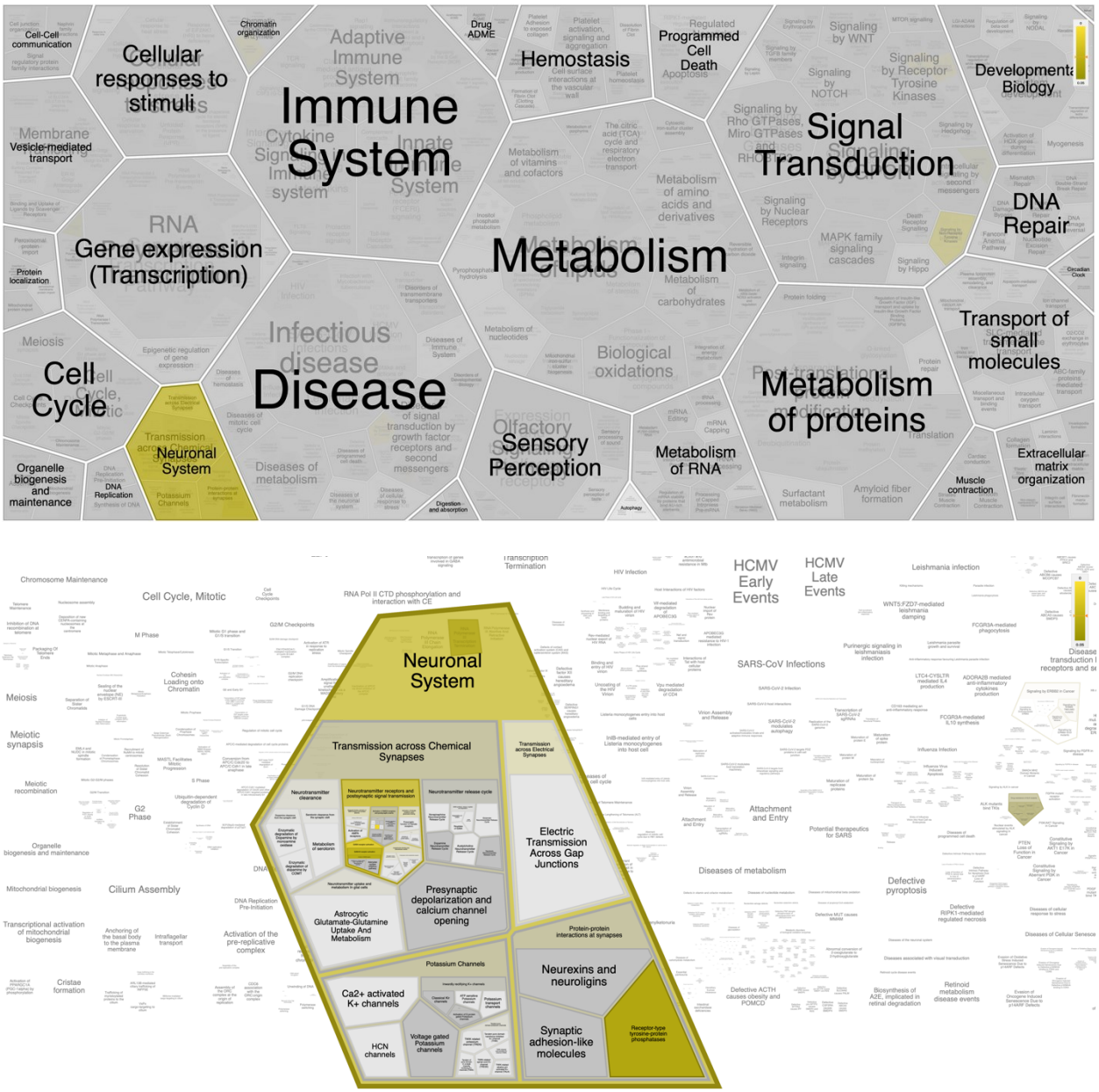

Figure 4.27: Voronoi diagram of pathways. A voronoi diagram of the overrepresented pathways analysis according to p-value is detailed. On top is the overall view, and below is the detail of the neuronal system. (https://reactome.org).

After performing a bioinformatic analysis of the pathways involved, we decided to prioritize the 473 candidate genes according to two main criteria: genes encoding for proteins expressed in the central nervous system and genes involved in the metabolism or neurotransmission of first- and second-generation antipsychotic drugs.

Human protein-coding genes expressed in the central nervous system (CNS) are in total about 16507 and are considered the most represented class; of these, 2709 are classified as elevated in the brain, while 204 genes have been detected exclusively in brain tissue (**Figure 4.28**). According to The Human Protein Atlas database (https://www.proteinatlas.org) 103 genes out of a total of 473 identified, are expressed in brain tissue with high specificity.

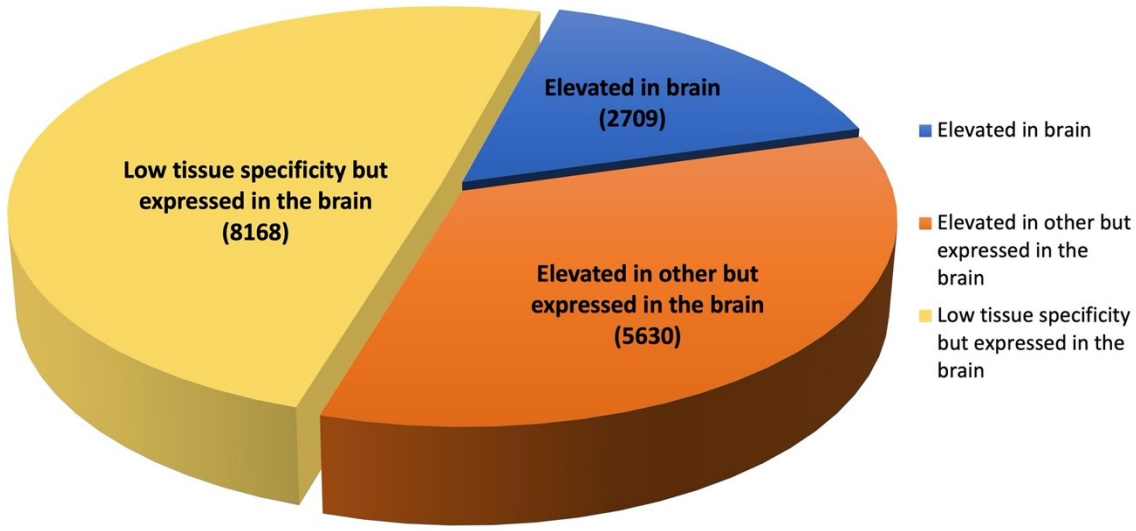

Figure 4.28: Summary of gene expression in the brain. The pie chart shows the number of partial genes, classified as elevated in the brain, elevated in other tissues but expressed in the brain, and low tissue specificity but expressed in the brain. The partial numbers, added together, make 16507, the total number of genes expressed in the brain.

Subsequently, we performed a selection of candidate genes according to more stringent established criteria inherent to the unique characteristics of these 11 NMS patients analyzed in this second part of the study.

We selected a total of 52 candidate genes belonging to one of the pathways involved in neurotransmission, including neurotransmitter receptors (dopamine, serotonin, or muscarine neurotransmission), the profile of antipsychotic drug receptor targets, downstream signaling proteins, transporters, and genes involved in antipsychotic metabolism (**Table 4.25**). These genes were selected as a result of extensive bioinformatic analyses conducted through DrugBank (https://go.drugbank.com), PubChem (https://pubchem.ncbi.nlm.nih.gov), STITCH databases (http://stitch.embl.de) or based on results obtained from previous association studies on APs (Siafis et al., 2018). Finally, the various data obtained from the different research, were cross-referenced to obtain a list of drug target genes taken by NMS patients.

		Haloperidol	Promazine	Promethazine	Zuclopenthixol	Aripiprazole	Quetiapine	Risperidone	Clozapine	Olanzapine
Dopamine	DRD1	+			++	+	+	+	+	+
	DRD2	+++	+++	+	++	+++	+++	+++	+++	+++
	DRD3	+				+	+		+	+
	DRD4					+++	+		+	+
	DRD5				+	+	+			+
Histamine	HRH1	+	+	++	+	++/-	+	+	+/-	+
	HRH2			+		+				
	HRH3					+				
	HRH4					+			+/-	
Serotonine	HTR1A					+++	+	+	+	+
	HTR1B					+	+		+	+
	HTR1D					+	+	+	+	+
	HTR2A	+	+	+	+	++	+++	+++	+++	+++
	HTR2B					+				
	HTR2C	+	+			++	+	+	+	+
	HTR3A					+	+		+	+
	HTR1E					+	+		+	+
	HTR1F									+
	HTR5A					+				
	HTR6					+	+		+	+
	HTR7					++	+	+	+	
Alpha-adrenergic	ADRA1A		+	+	+	+/-	+	+	+/-	+
	ADRA1B			+		+/-		+	+/-	+
	ADRA1D	+	+	+			+	+	+	+
	ADRA2A			+	+	+/-	+		+/-	
	ADRA2B			+		+/-	+	+	+/-	
	ADRA2C			+		+/-	+	+	+/-	
Muscarin	CHRM1		+	+		+	+		+/-	+
	CHRM2			+		+	+		+/-	+
	CHRM3			+		+	+		+/-	+
	CHRM4			+		+	+		+/-	+
	CHRM5			+		+	+		+/-	
Beta-adrenergic	ADRB1					+				
	ADRB2					+				
Metabolism	CYP1A1	+							+	
	CYP1A2	+	+						+	+/-
	CYP2A6									
	CYP2B6			+					+	
	CYP2C8								+	
	CYP2C9	+	+	+					+	+/-
	CYP2C19	+	+				+		+	+
	CYP2D6	+	+	+	+	+	+	+	+	+/-
	CYP3A4	+	+		+	+/-	+	+	+	+
	CYP3A5	+				+	+			+
	CYP3A7	+				+	+			+
	CBR1	+								
	UGT1A4								+	+/-
	UGT1A9	+								
Transporter	FMO3								+	+
	ABCB1	+		+			+	+	+	+
	ABCC3			+						
	ABCC4			+						

Table 4.25: Receptor and metabolic binding profile of antispychotics. The degree of binding of antipsychotics to receptors, metabolic enzymes, and transporters is shown in detail in the table. Data taken from Drugbank, PubChem, STITCH and (Siafis et al., 2018). The number of crosses is correlated to binding affinity. +/-: No pharmacological action.

Therefore, the list of 473 genes mapped to structural variant regions shared among all 11 NMS subjects was cross-referenced with the list of 52 genes identified on the basis of neurotransmitter receptor interaction, receptor-binding targets profile or metabolism of antipsychotic drugs taken by the patients (**Table 4.25)**. As a result of this further analysis, two candidate genes were identified: the adrenoceptor alpha 1B gene (*ADRA1B*), belonging to the adrenoreceptors group of 9 genes and member of the G protein-coupled receptor superfamily, and the dopamine receptor D_3 gene (*DRD3*), widely described as a target of antipsychotic drugs, especially second-generation SGAs. This receptor is located within the limbic areas of the brain, which are associated, among others, with cognitive and emotional functions. In addition, both of these two genes are widely expressed in the brain, and both belong to the list of 103 genes expressed in brain tissue with high specificity according to The Human Protein Atlas database (https://www.proteinatlas.org) (**Figure 4.29** and **4.30**).

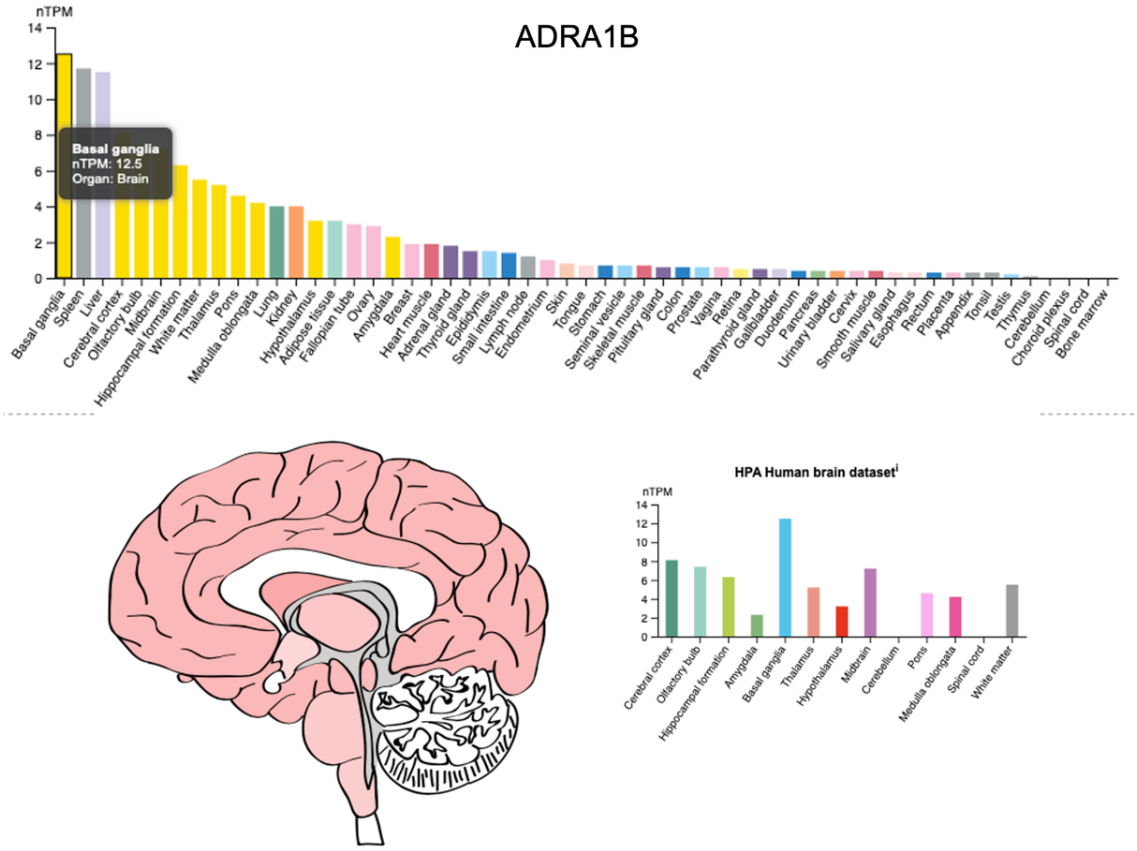

Figure 4.29: *ADRA1B* RNA and protein expression. Protein and RNA expression for *ADRA1B* is shown for all tissue in the bar plot. *ADRA1B* RNA expression in the main human brain regions is also shown (https://www.proteinatlas.org).

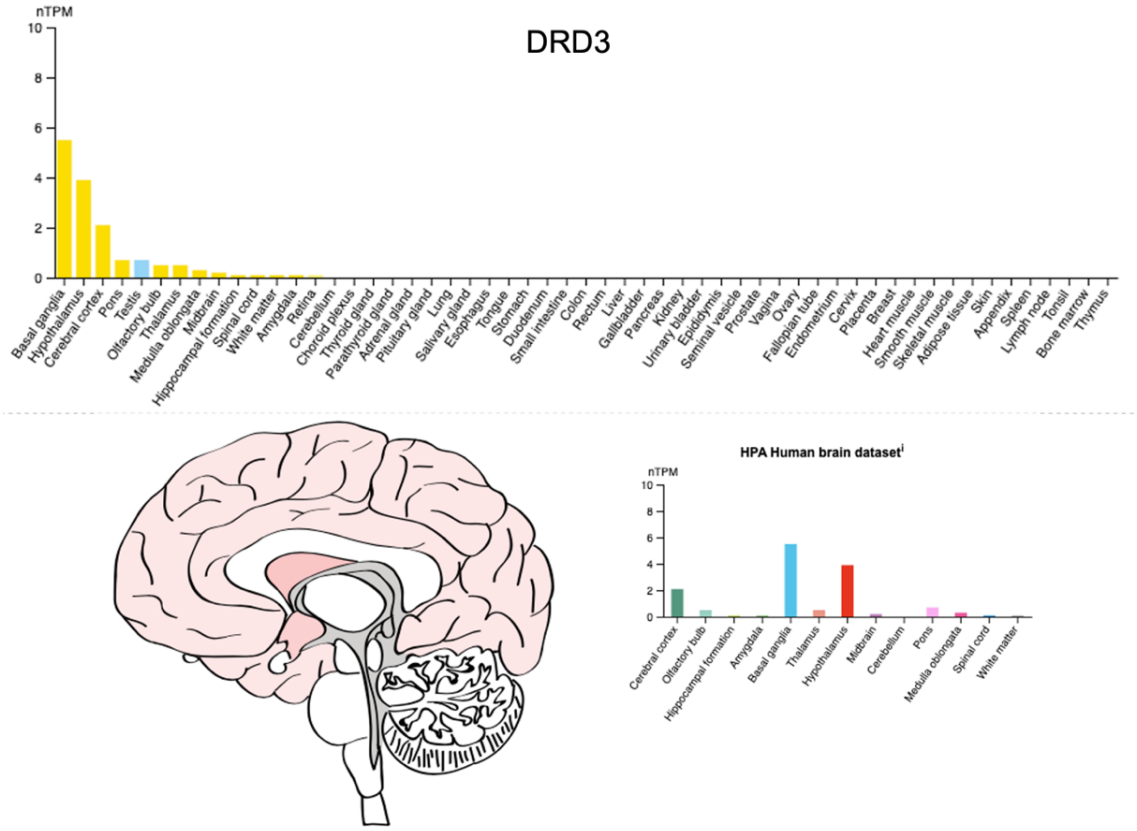

Figure 4.30: DRD3 RNA and protein expression. Protein and RNA expression for DRD3 is shown for all tissue in the bar plot. DRD3 RNA expression in the main human brain regions is also shown (https://www.proteinatlas.org).

The structural variant that affects *ADRA1B* is an insertion of a strand of nucleotides in place of a double AA, in heterozygosis, that affect the upstream 5' region of the gene (in position 5:159893295-159893297).

The structural variant that affects the *DRD3* gene is configured as a small deletion of 327 bp in homozygosis that overlaps with the promoter region of this gene. In this interval no structural data have been reported yet (**Figure 4.31**). Therefore, this deletion within the *DRD3* gene could be a peculiar hallmark among our cohort of NMS patients, and could lead to misregulation of protein, or even a complete lack of the protein product from this gene.

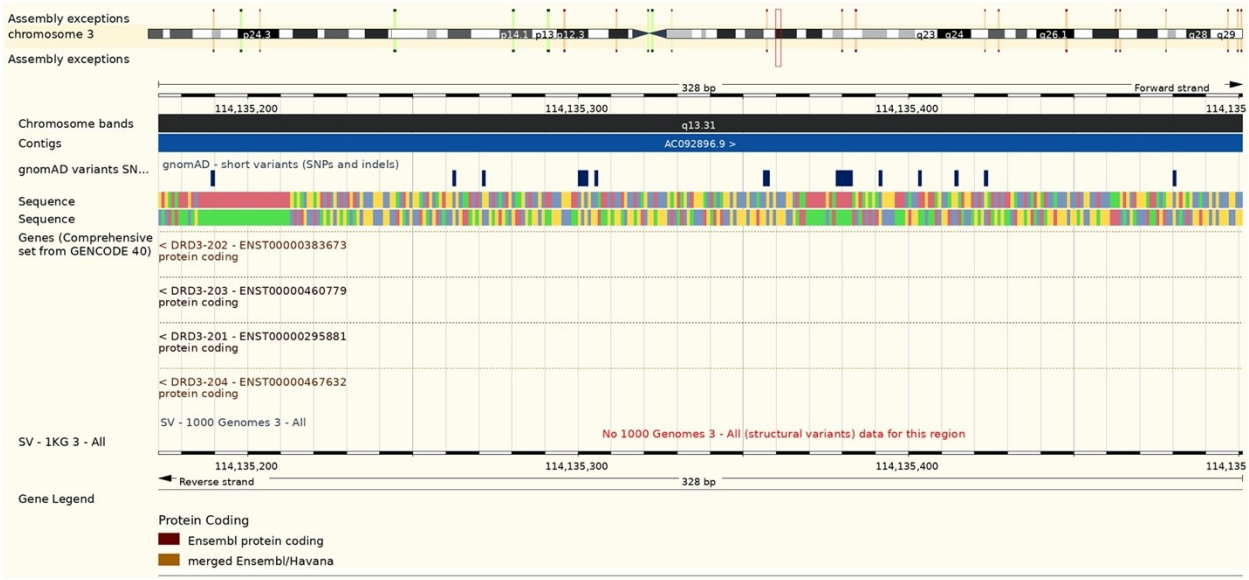

Figure 4.31: Genomic interval of SV on chr3. In the figure is detailed the genomic location of the 327bp structural variant deletion at position chr20q13.31 affecting *DRD3* gene. No structural variants are reported in this region (https://www.ensembl.org/index.html).

In conclusion, in-depth analysis shows that most of the 1024 SVs shared among the cohort of all 11 NMS patients were affecting protein coding sequence, where 473 genes were mapped. By further filtering these genes for proteins expressed in the CNS and genes involved in the metabolism or neurotransmission of antipsychotics, we were able to identify two structural variants, one insertion in heterozygosity and one deletion in homozygosity, affecting the adrenoreceptor alpha 1B (*ADRA1B*) gene and the gene coding for dopamine D3 receptor (*DRD3*), respectively, possible candidate genes for susceptibility to NMS.

5 DISCUSSION AND CONCLUSION

Neuroleptic malignant syndrome (NMS) is an infrequent but severe and possible life-threatening adverse reaction, that can occur in associations with dopamine antagonist drugs treatment, including first- and second-generation antipsychotics usually prescribed to patients with psychotic disorders or psychosis, or the sudden withdrawal of treatment with dopamine agonists medications, with which Parkinson's patients are typically treated (Caroff and Mann, 1993; J. R. Strawn, Keck and Caroff, 2007; Pileggi and Cook, 2016).

Environmental temperature, physical stress and others are risk factors that may concur to the onset of the syndrome. NMS is a diagnosis of exclusion, and other disorders shares symptoms with this syndrome. Therefore, the differential diagnosis of NMS is both complicated and challenging.

The precise pathophysiology underlying NMS remains largely uncertain and unpredictable, so far. Dysregulation of the central dopaminergic leading to a reduction or a blockade of the D_2 receptor seems to be associated with the onset of the syndrome (J. R. Strawn, Keck and Caroff, 2007; Berman, 2011; Mi *et al.*, 2011; Velamoor, 2017).

Few studies conducted mainly on a Japanese cohort of NMS patients have investigated with inconclusive and conflicting results the potential genetic predisposition that links polymorphisms of the dopamine receptor coding gene, DRD2 to the development of NMS (Kawanishi, 2003).

The present study was conducted on NMS patients of European non-Finnish ethnicity by performing whole genome sequencing with the aim of uncovering potential genetic traits associated with NMS susceptibility. In addition, three SNPs (rs1800497, rs1799732 and rs1799978) previously analyzed in the Japanese cohort were also investigated. Very restrictive criteria were applied to enrol NMS patients, particular attention was dedicated to the triggers: only patients who developed the NMS by administration of FGAs (10 patients) and SGAs (13 patients) were admitted to the study.

Of the three genetic polymorphisms analyzed, the rs1799732 SNP (also known as -141C Ins/Del) located in the promoter region of the *DRD2* gene was statistically associated with NMS susceptibility ($p<0.05$). The odds ratio estimated by logistic regression model resulted in a 5.5-fold times greater risk of developing NMS for patients carrying at least one G allele (G/G homozygote or G/GG heterozygote genotypes, namely G-carriers, over the GG/GG homozygote genotype, namely G-non carriers. Allele frequency of the G allele raised from f=0.072 frequency of the healthy control individuals (9 out 70) (gnomAD MAF (minor allele frequency) is 0.076 for the southern European population) to f=0.412 allele frequency of the NMS patients (7 out 17). Notably, in the 85.5% of the G-carrier NMS patients (6 out of 7) the trigger was a SGA, whereas

the FGA trigger was administered to 10 out of 17 NMS patients with GG/GG genotype, corresponding to 58.8%.

Therefore, the rs1799732 genetic variant may be considered a putative biomarker associated with an increased risk of developing NMS following administration of antipsychotic therapy. However not confirmed by in vivo PET (Jönsson *et al.*, 1999; Pohjalainen *et al.*, 1999; Hirvonen *et al.*, 2009), a study conducted by Arinami's group through a transactivation assay of a reporter gene revealed that variation of the genomic sequence of the promoter region of the D2 receptor (*DRD2*) given by the presence of the -141C deletion (G allele) led to a decrease in promoter strength and thus affected the expression or regulation of the gene (Arinami et al., 1997). These findings reinforce the theory that a blockade of the dopamine D_2 receptor, probably at the level of the signal transduction cascade, is one of the main and most important mechanisms underlying the onset of NMS (Henderson and Wooten, 1981; J. R. Strawn, Keck and Caroff, 2007; B D Berman, 2011; Velamoor, 2017). Indeed, dysregulation of D_2 receptor-mediated dopaminergic pathways has also been associated with schizophrenia, bipolar disorder and Parkinson diseases (Mi *et al.*, 2011). Finally, recent studies have indicated an association between the -141C Ins/Del and response to antipsychotic drugs, showing that individuals carrying the rarer Del allele had a poor response to treatment with antipsychotic drugs (Zhang, Lencz and Malhotra, 2010; Matsumoto *et al.*, 2018).

The presence of the G-allele of the rs1799732 SNP increases the risk of developing NMS in patients and it should be carefully considered by physicians before starting an antipsychotic therapy, in particular with SGAs. Further functional analyses will be performed to better understand the role of rs1799732 and the molecular events that lead the presence of this variant to NMS susceptibility.

Therefore, in this PhD thesis, a Whole Genome Sequencing was performed with the aim of identifying genomic traits such as CNVs (Copy Number Variation) and/or SVs (Structural Variants) shared among all patients that might be associated with NMS predisposition..

A total of 11 well characterized NMS patients (5 female and 7 male) were selected for WGS analysis by applying even more stringent criteria: the presence/absence of the rs1799732 variant and the trigger and treatment (mono- vs multi- therapy). Thus, NMS patients were sub-grouped in those who received a monotherapy of FGA (mono FGA: 4 patients) and those who were treated with SGA monotherapy (mono SGA: 5 patients); In addition, 2 patients were treated in two consecutive period with only one antipsychotic a time, FGA or SGA therapy (mono FGA + mono

SGA: 2 patients). Moreover, 7 were G-carriers and 4 were G-non carriers for the variant rs1799732.

CNVs are generally characterized by a loss or gain of genomic portions, which may be due to structural modifications within the genome, including deletions, insertions, duplications and sometimes even triplication, quadruplication and so forth, of smaller or larger genomic fragments, ranging from one kilobase (kb) to several megabases (Mb) in size, and also unbalanced translocations and inversions (Freeman *et al.*, 2006; Zhang *et al.*, 2009; Shaikh, 2017). Usually, certain CNVs termed "benign" are commonly diffuse throughout the genomes belonging to healthy subjects that do not seem to have any apparent association with any disease or pathological phenotypes. Nevertheless, numerous *de novo* or inherited CNVs have been associated with the development of a wide range of human diseases, defined copy number variation syndromes and disorders, that result from dosage disequilibrium of genes due to genomic rearrangements leading to gain or loss of genetic material. These CNVs syndromes and disorders may involve conditions affecting the neurological spectrum, such as intellectual disability (ID), neurodevelopmental abnormalities, neurodegenerative and neuropsychiatric disorders (including schizophrenia and autism spectrum disorders), as well as cancer, immune deficiency and multiple congenital anomaly syndromes. Therefore, CNVs are significantly impacting human health and medical diseases (Zhang *et al.*, 2009; Shaikh, 2017).

In each subject analyzed, approximately 800-900 CNVs (650 gains and approximately 200 losses) were found on average. Therefore, these CNVs were evaluated and compared with each other, extracting structural changes and shared traits, with the aim of identifying a shared pattern among all 11 NMS patients and among the various subgroups identified (G-carrier, G-noncarrier, FGA, and SGA patients).

As a result of this analysis, only a small number of CNVs (7 regions in total) were found to be shared among all NMS patients analyzed. To find out whether these 7 identified CNVs were already described in patients or in association with pathologies, or whether they might be a peculiar trait in common among our NMS patients, we consulted the database "DECIPHER" (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) (https://www.deciphergenomics.org) (Firth *et al.*, 2009).

Next, we proceeded with a bioinformatic analysis through the Ensembl database (https://www.ensembl.org/) using data collected in the 100 Genome phase 3 project and DGVa (Database of Genomic Variants archive, https://www.ebi.ac.uk/dgva/) to determine whether these

CNVs, were commonly frequent and therefore definable as a typical trait of the European population, or rather typical of these subjects. Of the 7 CNVs considered, only 2 were frequently present in the European population.

Because CNVs can affect the dosage of genes that drive differential levels of gene expression or a loss of coding functions, we studied and analyzed more in detail what the loss or gain of these genomic regions, shared among all patients, could affect and represent. Some identified CNVs have been mapped to centromeric regions or chromosomal bands in which genes or regulatory regions have not yet been described or mapped; therefore, although information on the presence of structural variant data has not yet been found in these regions, it cannot be ruled out that these CNVs may have a regulatory or structural function not yet known and reported.

Although affected by many gains described with high frequency in the European population (about 88%), in one of the shared regions among all NMS patients was found the presence of a miRNA (*MIR3680-1)*, which would seem interesting. miRNAs are a broad category of small endogenous, single-strand, non-coding RNAs that regulate and modulate gene expression (miRNA target genes) and can be involved in human diseases, for example neurological and neurodevelopmental disorders, cardiovascular pathologies and, mostly, cancer, since approximately 70% of miRNAs are expressed in the nervous system, and are therefore involved in neurodevelopment, neurotransmission, as well as dysregulation at the neurological level (Kosik, 2006; Qiao *et al.*, 2013).

Copy number variations are known to have an influence on miRNAs, miRNA's function and their role regarding the onset of human pathologies, although the precise effect of genomic CNVs on miRNA role is mostly unknown. Indeed, the association of CNVs and miRNA expression has been investigated in individuals with cognitive delay only in limited circumstances.

MIR3680-1, along with *MIR3680-2,* belongs to the MIR3680 MicroRNA family. *MIR3680-1* gain is reported as a *de novo* CNV with very high frequency (75%) while the remaining 25% is defined as uncertain inheritance. The gain of this miRNA has been reported in subjects who manifested, among other things, autistic behavior, intellectual disability (ID), abnormal cortical gyration, delayed speech and language development, global developmental delay and neurodevelopmental delay.

Although these results should be considered preliminary, a systematic analysis of the expression profile and function of miRNAs, in addition to the genes encoding CNVs, could be essential to discover new causes of susceptibility to the development of NMS.

In some of the identified CNVs, a number of pseudogenes and long-non-coding-RNAs (lncRNAs) have been identified, mostly unannotated or unprocessed, for which information on their frequencies and functions is not yet available in online databases.

Pseudogenes are copies of genes that have lost their coding function. Around 27000 of ubiquitous pseudogenes are estimated in human genome. Even though evidence on their function is still debates, it is well known that some pseudogenes are essential elements of gene regulation of their respective genes. Moreover, many pseudogenes are also transcribed into small RNAs that interfere with the cellular concentration of miRNAs, regulating tumor suppressors and oncogenes (Tutar, 2012).

The *CCNYL3* is a pseudogene that positively regulates the activity of cyclin-dependent protein serine/threonine kinase. *CCNYL3* belongs to the cyclin family in which 31 genes are involved (https://www.genenames.org). Cyclins are a family of proteins regulate cell cycle progression by forming a complex with the enzyme CDK (cyclin-dependent kinase). The variation of cyclin concentration depends on gene expression, therefore a loss of genetic portion in which this gene is localized probably leads to a down-regulation of the cell cycle, although there are not yet information present in databases that can confirm or deny this theory.

However, findings collected over the past years demonstrate that lncRNAs are extensively expressed in the human genome and also important elements in gene regulation.

lncRNAs are estimated to be more than 16000, although some approximations would amount to 100000, and are defined as RNAs longer than 200 nucleotides that originate from genome transcription but are not translated into functional proteins. Even though the evidence on their function and number is still debated, it is known that some lncRNAs, whose expression appears to be regulated, are widely expressed in the human genome and perform important cellular functions and several gene regulatory mechanisms (Statello et al., 2021). Indeed, based on their localization and their ability to establish genome-specific interactions with DNA, RNA, and proteins, they have important cellular functions: they are able to regulate the assembly and function of membrane-free nuclear bodies and chromatin function, modifying the stability and translation of mRNAs, including splicing and translation, and influencing signaling pathways. They also have regulatory functions on the expression of many genes, near and far, influencing their transcription. These functions, are known to influence gene expression in a variety of biological process, and indeed in pathophysiological backgrounds, such as immune responses, cancer and neuronal disorders (Statello *et al.*, 2021). Thus, dysregulation of lncRNAs due to gains and losses of

genomic portions of DNA, can lead to malfunction of the lncRNA-target-genes they influence, and therefore lead to dysregulation of various systems and pathways, including the central nervous system. Furthermore, a combined action of various lncRNAs, could have several effects, altering the expression and functions of multiple genes in regulatory processes, resulting in a synergistic action either in association with each other, or in association with the rs1799732 variant, resulting in a pleiotropic effect.

Although these observations should be considered preliminary, our results nevertheless suggest that specific expression patterns of these functional lncRNAs are excellent potential biomarkers of disease and dysregulation of various gene functions, and thus should be given greater consideration and studied in depth as therapeutic targeting.

The only gene that has been found to be mapped within one of the 5 out of 7 infrequent CNVs shared among all patients, for which no variant gains or losses were reported in the database, is the potassium inwardly rectifying channel (Kir2.6) subfamily J member 18 (*KCNJ18*) gene.

The *KCNJ18* gene belongs to a group of 16 genes called "Potassium channel internal rectification subfamily J", a specific subset of potassium selective ion channels that are characterized by a greater tendency to allow potassium to flow into the cell rather than out of it. They are reported to be targets of numerous toxins, and channel malfunction has been implicated in several diseases.

KCNJ18, expressed predominantly in skeletal muscle, is transcriptionally regulated by thyroid hormone; the protein acts by maintaining resting membrane potential in excitable cells and helps repolarize cells after their depolarization. Findings suggest that variations in the *KCNJ18* gene may cause predisposition to episodes of thyrotoxic periodic paralysis (TPP) (Ryan *et al.*, 2010). TPP (OMIM: 188580) is a rare but threatening neurological disease, potentially lethal, characterized by acute and sporadic attacks of weakness and hypokalemia during the thyrotoxic state leading to paralysis. Mechanisms underlying hypokalemia are poorly understood. The predominant theories involve increased activity of the Na-K ATPase pump and the presence of mutations in genes encoding Kir channels in skeletal muscle (Ryan *et al.*, 2010; Siddamreddy and Dandu, 2022).

However, our hypothesis, that needs to be further investigated, is that the 3-fold gain of the 170 kb CNV containing the *KCNJ18* gene will likely lead to dysregulation of potassium channel Kir, which may lead to increased potassium concentration and possibly hyperkalemia. Extracellular potassium concentrations strongly influence cell membrane polarization, which in

turn influences important cellular processes, such as nerve impulse conduction and muscle cell contraction. Moreover, hyperkalemia is reported to be one of the manifestations of muscle damage and rhabdomyolysis, known as two of the major hallmarks and complications of NMS. (Mann *et al.*, 2003; Oruch *et al.*, 2017; Sahin *et al.*, 2017; Kansal *et al.*, 2019). These findings, in addition to identifying *KNCJ18* as a possible putative candidate gene for susceptibility to develop NMS, and therefore as a pharmacogenomic biomarker, may also be helpful to clinicians in explaining the still unclear pathophysiology underlying the development of NMS.

SVs are generally characterized by rearrangements of genomic portions, such as deletions and insertions, inversions, translations, and tandem repeats ranging from 50 base pairs (bp) to several megabases (Mb) in size, and also unbalanced translocations and inversions (Sharp, Cheng and Eichler, 2006). It has been estimated that these structural variants (SVs) affect approximately 800 genes and may influence more or less heavily the human phenotype. Many previous works published in the literature suggest a key role of these structural variants, along with copy number variations, in the biology of rare diseases and in modulating heritable gene expression variants in human population. Moreover, rare and de novo SVs have been studied in association with autism and schizophrenia disorders (Sharp, Cheng and Eichler, 2006; Stone *et al.*, 2008; Chiang *et al.*, 2017; Abel *et al.*, 2020). However, no studies have been conducted to date linking SVs to NMS susceptibility.

Of all the structural variants analyzed in our patient cohort, which averaged slightly more than 10000 per sample, 1024 (approximately 10%) were identified shared among all 11 NMS patients. First, we focused on those that could affect only the coding region, and thus have effects on protein assembly, structure, and function. Therefore, first we extracted the variants that had "protein coding" as the Biotype filter, and "high" as the Impact. Of these, only two SVs, one on chr 14 and the other on chr X, were identified as a result of the filtering procedure.

In the second instance we limited ourselves to filtering and extracting variants that had only "protein coding" as Biotype. Therefore, 435 structural variants were identified. In order to identify shared pathways that might be compromised in some way by the presence of these structural variants, we were able to estimate that 264 of the 473 identifying genes were found in at least one of the 1021 shared pathways identified through an extensive analysis conducted in Reactome (https://reactome.org). Most of the 264 genes identified are involved in disease (especially cancer), signal transduction, gene expression (transcription), chromatin organization, and the neuronal system pathways. Thus, significant structural changes in genes involved in these pathways, such

as those identified as a result of next-generation sequencing analysis, may affect not only protein expression, but also have a cascade effect on the underlying biochemical pathways.

Lastly, we decided to prioritize the 473 candidate genes according to two main criteria: genes encoding for proteins expressed in the central nervous system and genes involved in the metabolism or neurotransmission of first- and second-generation antipsychotic drugs. As a result of this further analysis, we were able to identify two structural variants located within as many genes: an insertion in heterozygosity and a deletion in homozygosity, affecting respectively the gene of adrenoreceptor alpha 1B (*ADRA1B*), and the gene coding for the dopamine D3 receptor (*DRD3*). Both of these two genes, in addition to being expressed in brain tissue with high specificity according to The Human Protein Atlas database (https://www.proteinatlas.org), also belong to the list of genes identified on the basis of the pharmacokinetics and pharmacodynamics profile of antipsychotic drugs.

The structural variant that affects *ADRA1B* is an insertion of a strand of nucleotides in place of a double AA, in heterozygosis, that affect the upstream 5' region of the canonical transcript of the gene (at 5:159893295-159893297 position). Alpha 1B adrenoreceptor (*ADRA1B*) is one of 3 subtypes of alpha-1 adrenergic receptors (alpha-1A, -1B and -1D), they are a class of G protein-coupled receptors that all signal through the Gq/11 family of proteins. They are targets of catecholamines, particularly adrenaline and norepinephrine, with which they bind to stimulate the sympathetic nervous system response. They are involved in the activation of mitogenic responses and regulate the growth and proliferation of many cells. At the pharmacological level, *ADRA1B* is the receptor target of mostly second-generation antipsychotics such as aripiprazole, risperidone, clozapine, olanzapine and the drug promethazine, a first-generation antihistamine often used medically as an antipsychotic medication, and also been associated with the development of NMS in treated patients. Although this receptor has never been studied in association with neuroleptic malignant syndrome, it is interesting to investigate as a putative susceptibility gene for NMS since it is involved in signal transduction pathways and in calcium signaling pathway. Moreover, adrenoceptors act as stimulatory receptors involved particularly of vascular smooth muscle and in local vasoconstriction, as well as in the control of blood pressure and temperature (Docherty, 2010). Therefore, minimal genetic alterations involving genes belonging to this class of receptors could cause disruption or dysregulation of the signal transduction cascade as well as be implicated in the abnormal response manifestations later due to the development of neuroleptic malignant syndrome.

The D_3 receptor, encoded by the dopamine receptor D_3 gene (*DRD3*) within the chromosome 3, is mainly present in the limbic system, more detailed in the *nucleus accumbens*, in the *substantia nigra pars compacta* and in the ventral tegmental area. The D_3 receptor, along with D_2 and D_4 receptors, belongs to the D_2–like subgroup and it is also thought to act as an autoreceptor (as well as D_{2S}) thereby regulating dopamine release from the presynaptic terminal; it also acts by signalling through G_i/G_o proteins, though not as efficaciously as the D_2 receptor. The D_3 receptor, similarly to the D_1 and D_2, is also thought to be involved in the regulation of emotion and cognition, reward mechanisms and motor control, contributing to the modulation of the D_2 receptor properties, albeit to a lesser extent.

At the pharmacological level, *DRD3* is the receptor target of one first-generation antipsychotic (haloperidol) and mostly second-generation antipsychotics such as aripiprazole, quetiapine, clozapine and olanzapine. Although this gene has never been studied in association with NMS, it has been reported that a missense variant located in a coding region of the *DRD3* gene, known as rs6280 or Ser9Gly because produces a substitution from serine (Ser) to glycine (Gly), has been shown to affect dopamine binding affinity and may contribute to individual differences in susceptibility to antipsychotic-induced tardive dyskinesia (TD); in addition, this variant appears to contribute to treatment variability, as carriers of the Ser9gly variant appear to have a better response to pharmacological treatment with antipsychotics (Bakker, van Harten and van Os, 2006; Arranz *et al.*, 2013). Furthermore, genetic differences in dopamine receptor function may influence changes in dopaminergic signaling that modulate emotion and motivation (Savitz, Colin A Hodgkinson, *et al.*, 2013).

The D_3 receptor is hard to investigate because of its low abundance and because there are not selective ligands for it. However, *DRD3*-deficient mouse models have been created through a gene targeting strategy in embryonic stem cells. The binding of iodosulpride (a dopamine antagonist) to D_3 receptors was lacking in mice homozygous for the mutation and significantly attenuated in heterozygous mice. Moreover, homozygous mice lacking D_3 receptors exhibited hyperactivity, increased locomotor activity and rearing behavior (Acilli *et al.*, 1996).

Therefore, it is possible to hypothesize that the deletion of 327 bp in homozygosis at the promoter region of *DRD3* gene, likely affects the expression of this gene encoding a dopamine receptor mainly expressed in the limbic system and associated with regulation of cognitive and emotional functions, reward mechanisms and motor control. The lack of the D_3 dopamine receptor in these regions of the brain could dramatically lower the dopamine binding capability of binding to its pharmacological target (dopamine antagonist). The resulting dysfunction of pharmacological

binding could lead the dopamine uptake near to a critical threshold or an interruption of the dopamine signaling cascade that could trigger the NMS adverse reaction development if the patient requires a dopamine antagonist therapy with antipsychotics medication.

In conclusion, this evidence seems to suggest that there are a number of potentially candidate genes, two of which encode for dopaminergic receptors, one for an adrenergic receptor and one for a potassium channel, that appear to be strongly involved in susceptibility to the development of NMS, and thus should be further studied regarding the manifestation of the syndrome. Furthermore, more in detail, it seems that genomic variations involving dopamine D_2 and D_3 receptor genes are related to the early developmental stage of the syndrome, probably due to a downregulation or a blockade of the dopamine signalling cascade in the central nervous system, whereas genetic variations affecting the alpha 1B adrenoreceptor (*ADRA1B*) and the potassium inwardly rectifying channel gene (*KCNJ18*) would instead appear to explain the genetic mechanisms involved in the typical clinical manifestation of the neuroleptic malignant syndrome's response.

Thus, the genetic variants discovered in this study are candidate markers of NMS to be deeply investigated by functional assays, meanwhile these genetic markers should be evaluated in clinical practice for tailoring antipsychotic treatment with the goal of maximizing efficacy and reducing adverse drug reactions

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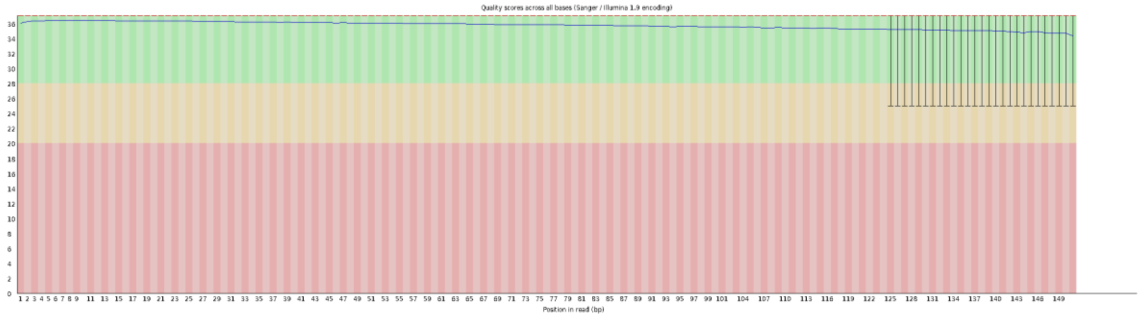
8 APPENDIX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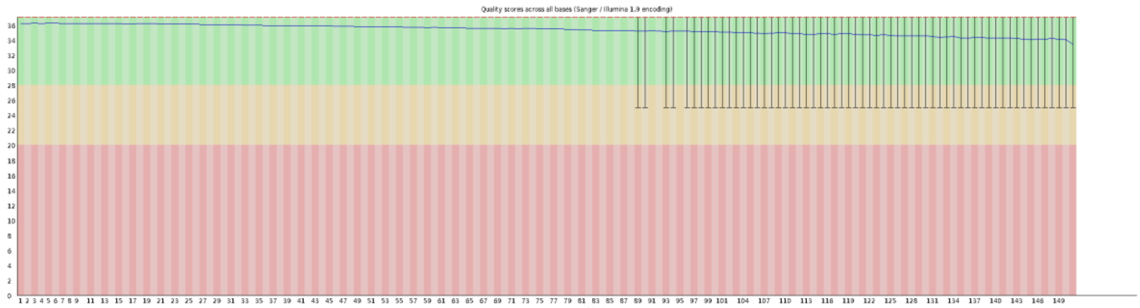
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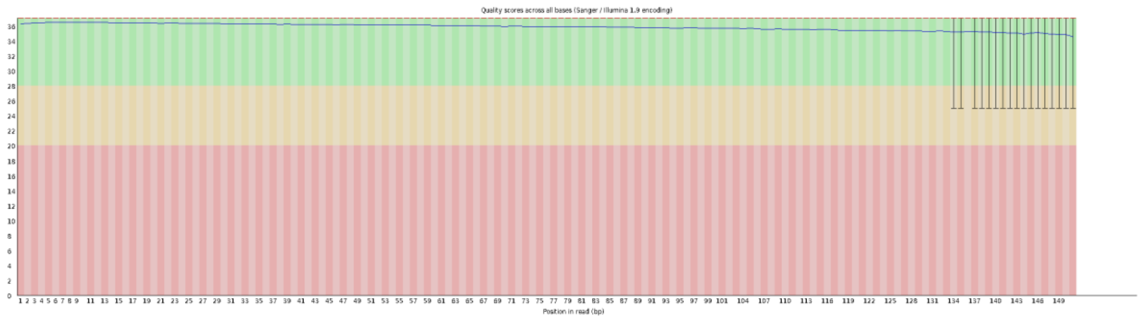
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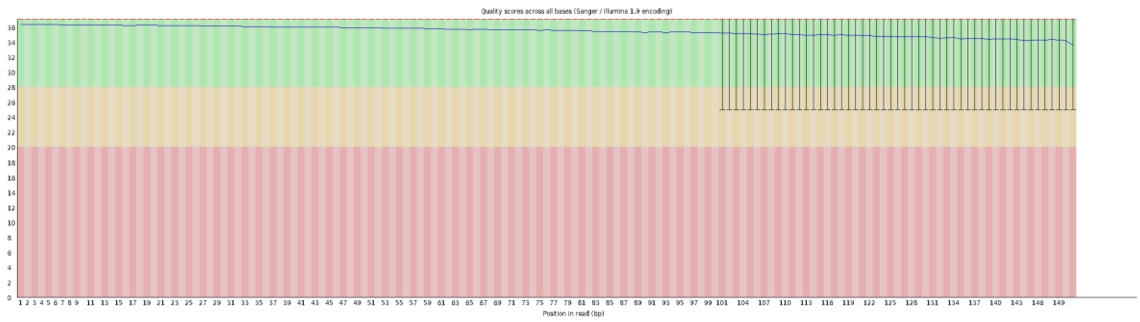
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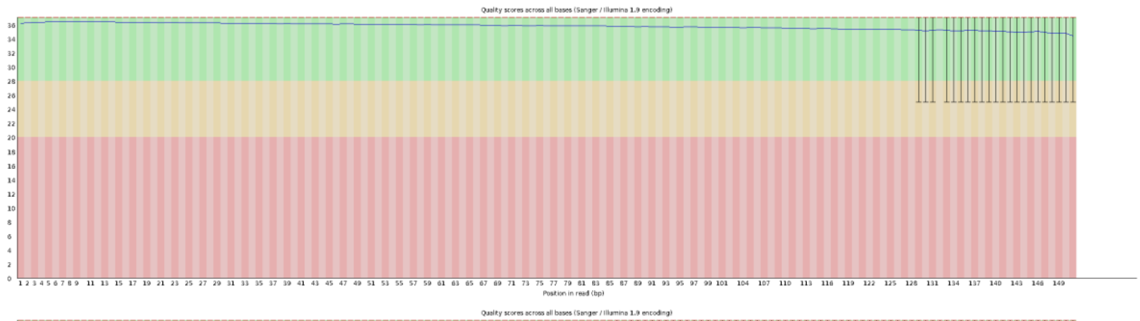
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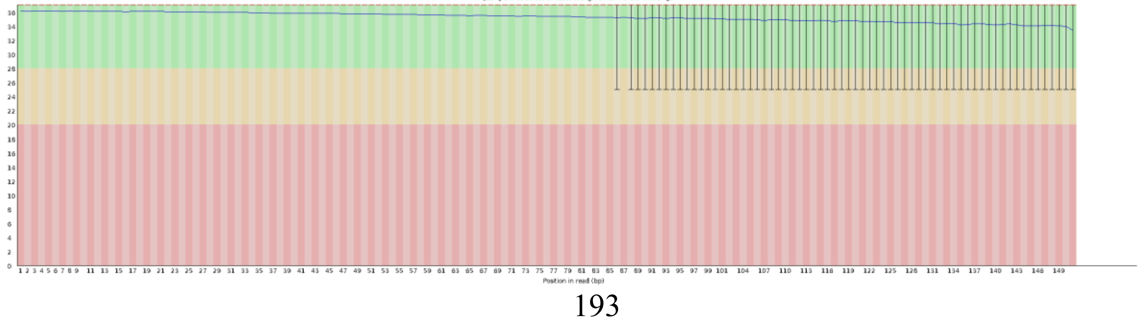
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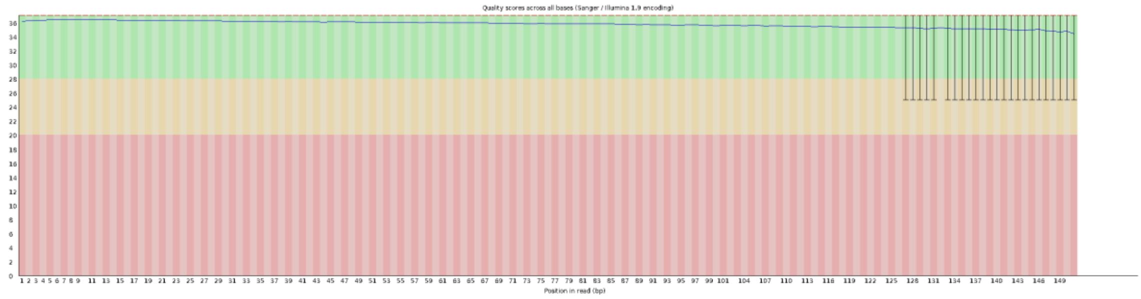
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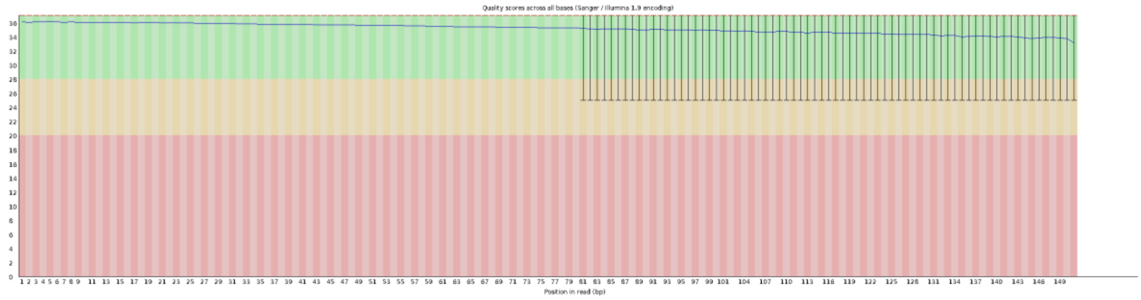
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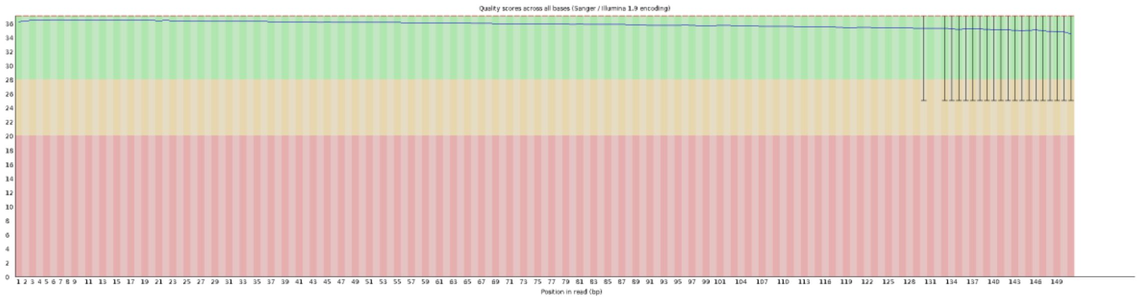
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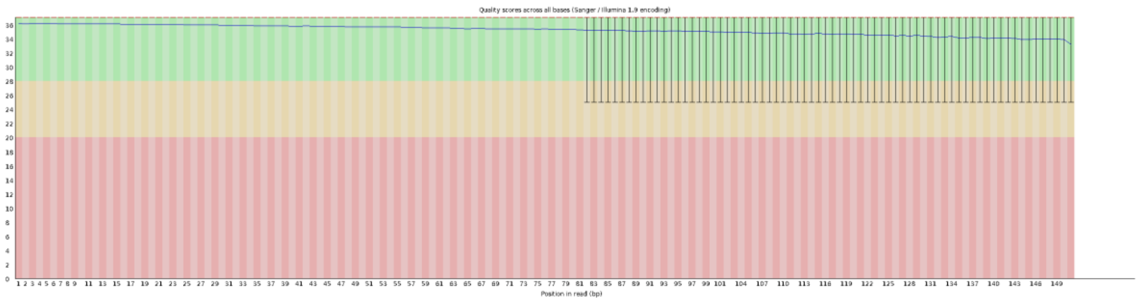
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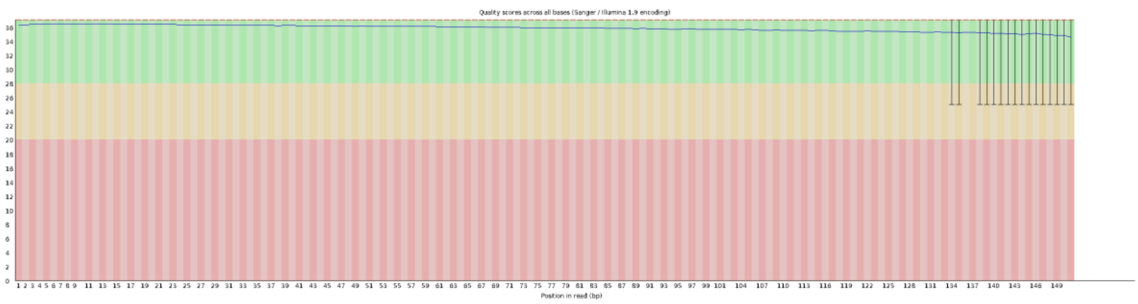
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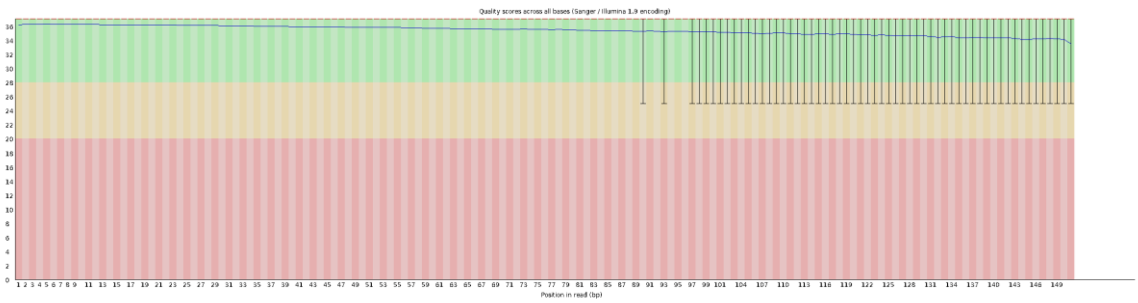
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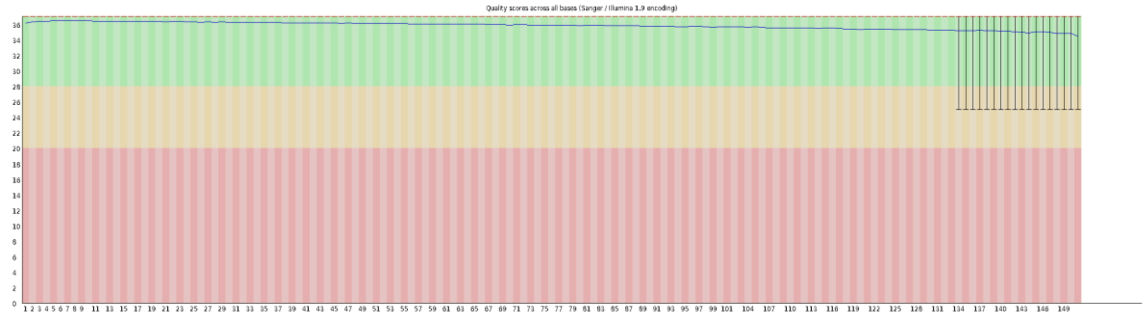
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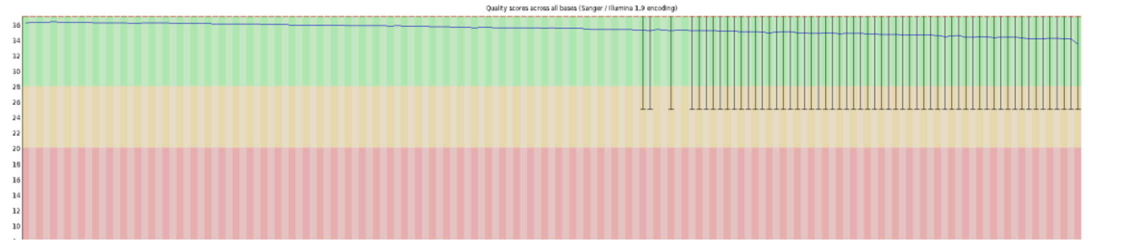
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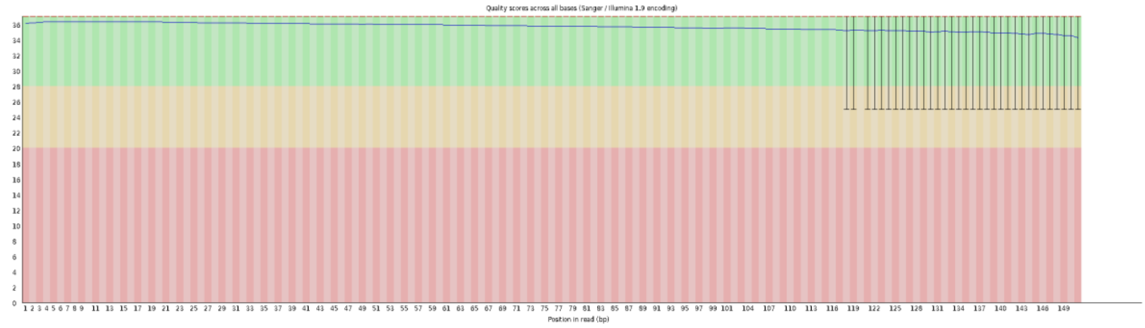
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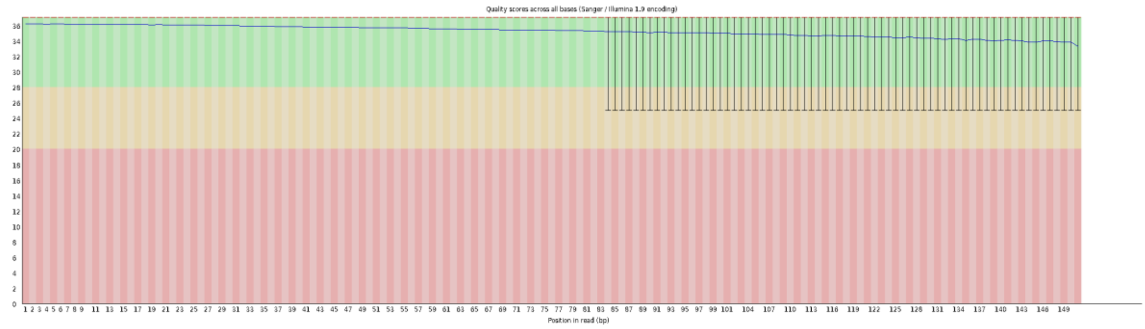
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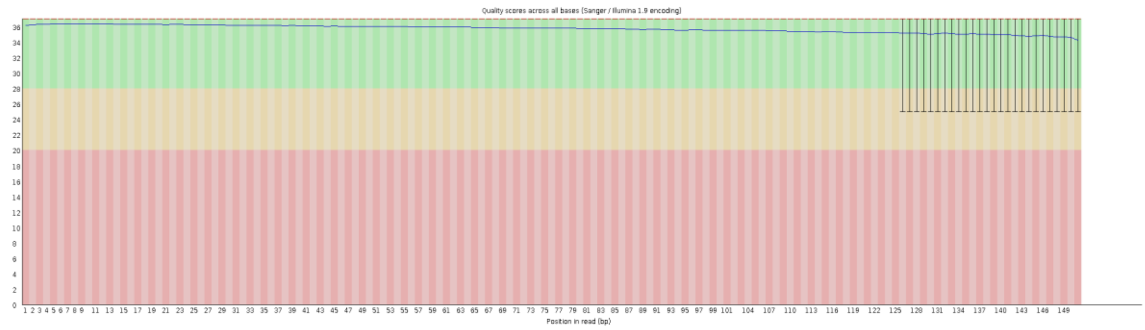
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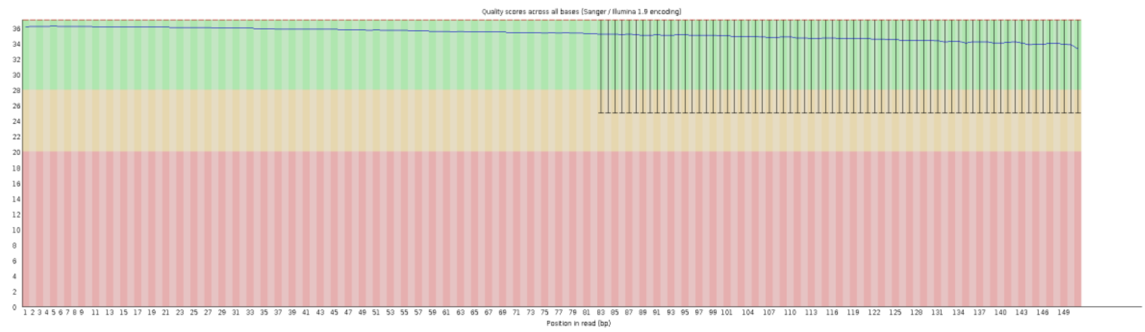
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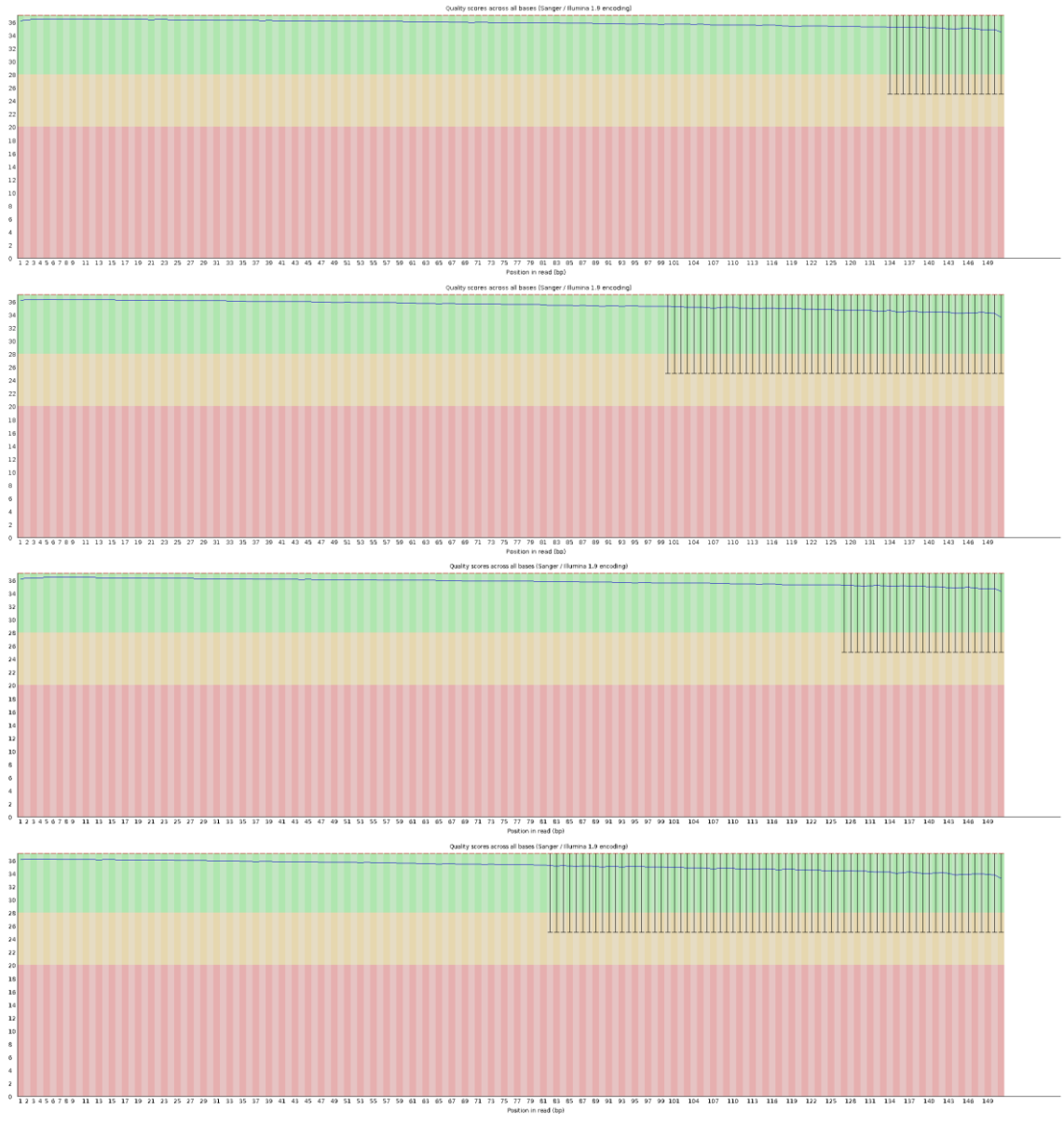
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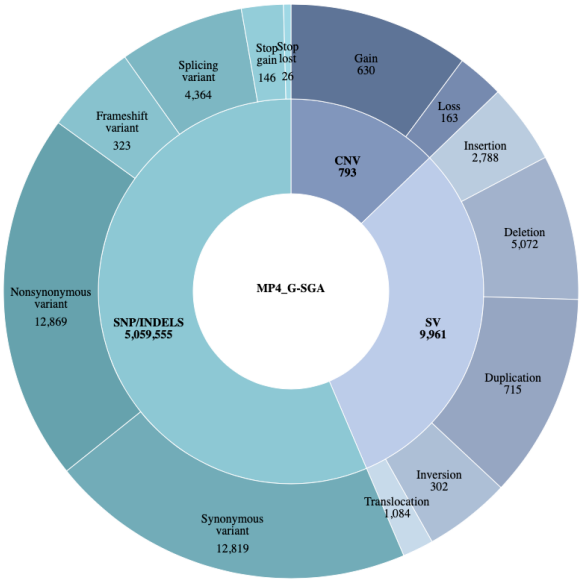
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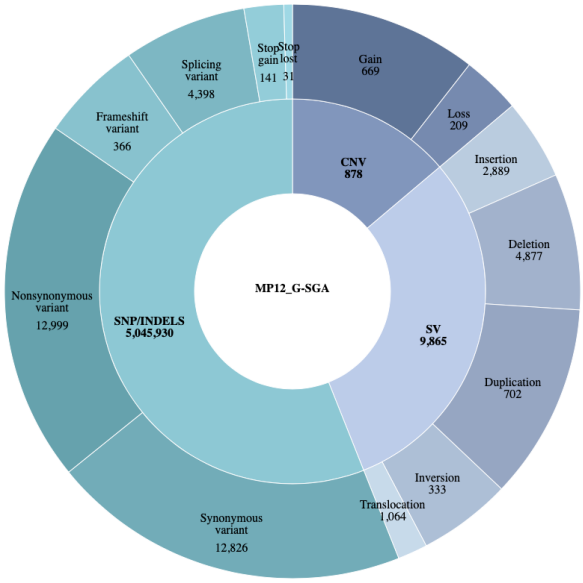
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Stop lost
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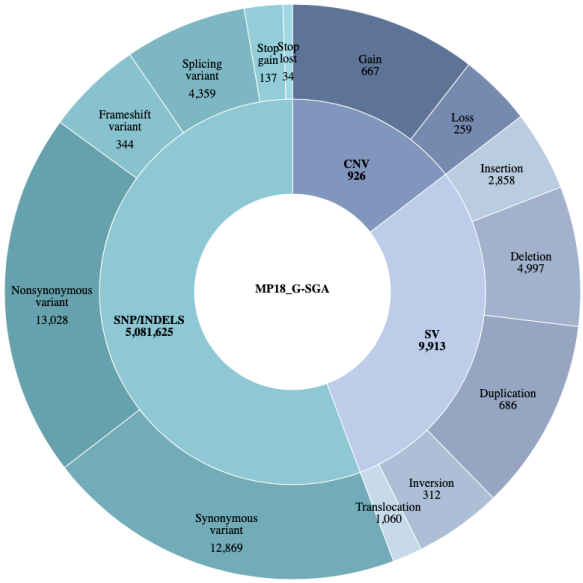
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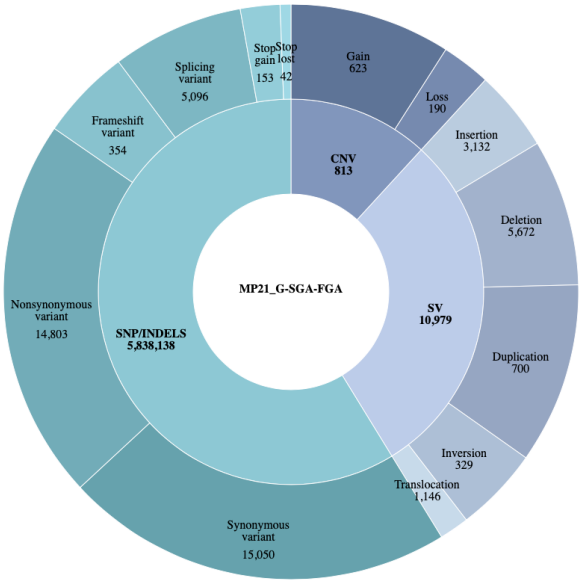
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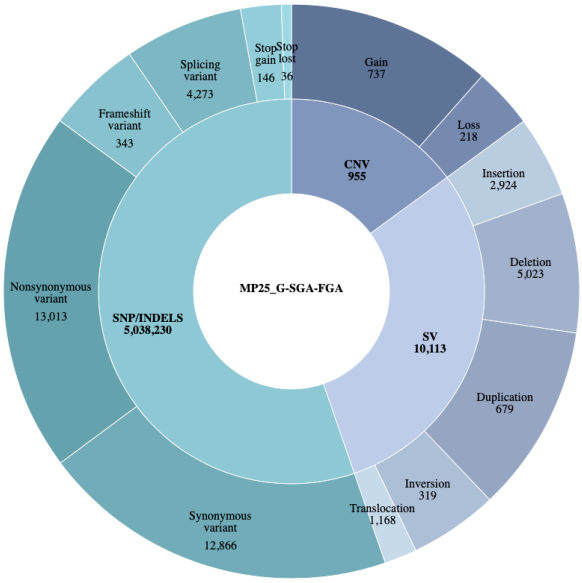
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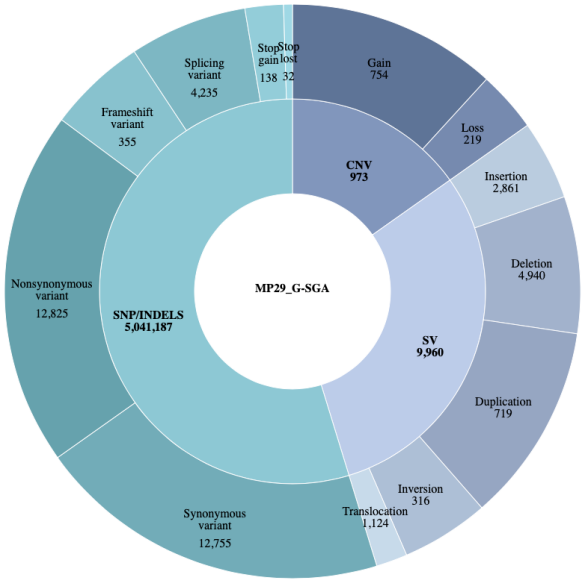
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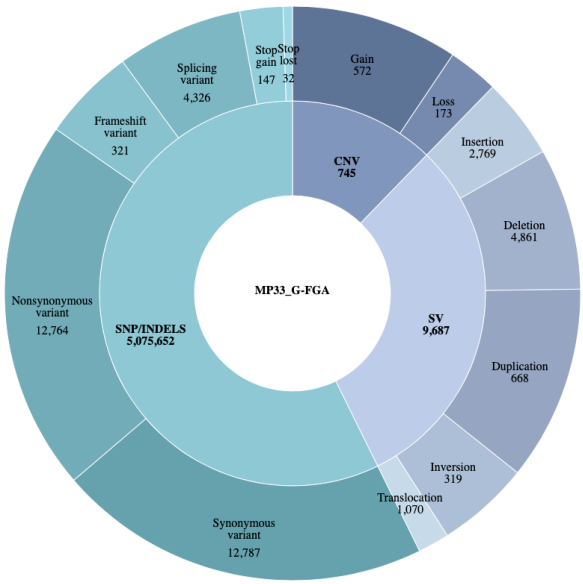
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5,058,555
Gain
660
Loss
181
Insertion
2,680
Deletion
4,796
Duplication
637
Inversion
329
Translocation
1,112
Synonymous
variant
12,725
Nonsynonymous
variant
12,667
Frameshift
variant
347
Splicing
variant
4,340
Stop
gain
146
Stop
lost
35

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