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Identification of a genetic variant of the *DRD2* promoter associated to Neuroleptic Malignant Syndrome

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ABSTRACT

Neuroleptic Malignant Syndrome (NMS), Serotonin Syndrome (SS) and Malignant Hyperthermia (MH) are severe adverse reactions to drugs such as antipsychotics, serotonergic agents, halogenated anesthetic gasses or succinylcholine, characterized by hyperthermia resistant to pharmacological treatments with antipyretics and non-steroidal anti-inflammatory drugs. These diseases are often associated with muscle stiffness, rhabdomyolysis, altered mental status, sympathetic nervous system lability and other shared clinical signs.

The identification of the triggering agents plays a major role in differential diagnosis. Patients are often treated with drug cocktails of antipsychotics and serotonergic antidepressants simultaneously, making difficult the differential diagnosis between NMS and SS. Furthermore, clinical manifestations with characteristics of both SS and NMS can be triggered by substances of abuse such as 3,4-methylenedyoximethamphetamine (MDMA) and cocaine.

It is generally accepted that blockade of dopamine D_2 receptor may be a critical step for the onset of NMS. Given similarities in clinical presentation, common underlying biochemical mechanisms involving dopamine D_2 receptor have been proposed. Findings in pharmacogenetics indicate that genetic polymorphisms are associated with the interindividual differences in drug responses concerning both efficacy and adverse reactions. Genetic markers of susceptibility to NMS in the D_2 receptor coding gene (*DRD2*) have been investigated on Asian individuals with conflicting results.

The present study was carried out on a cohort of Southern European non-Finnish individuals to investigate genetic markers of *DRD2* gene for susceptibility to NMS and NMS-like symptoms, triggered by antipsychotics and abuse of illicit drugs, respectively.

Among the analysed variants, rs1799732, mapped in the regulatory region of the *DRD2* gene, showed a statistically significant association with NMS susceptibility. Our results indicated that subjects carrying G/GG or G/G genotypes, namely G-carriers, of this variant showed 7.07-fold greater risk of developing NMS upon antipsychotic therapy.

More in detail, the second-generation antipsychotic aripiprazole and the first-generation antipsychotic haloperidol were the causative agents mainly

involved in triggering NMS in this cohort. These two antipsychotics were overall involved in triggering NMS in 11 out of 12 patients (91.6%), although with an opposite association with rs1799732 genotype, with aripiprazole mainly associated to G/GG genotype (80%), and haloperidol to GG/GG genotype (57%). Other risk factors commonly described for NMS were observed in 11 out of 12 patients, although no correlation with a specific genotype was found.

The NMS-like group of patients, whose adverse reactions were triggered by abuse of illicit substances, showed no association with this SNP. Thus, the variant rs1799732 might be a biomarker associated to antipsychotic drugs trigger and not with illicit substances, suggesting that different biochemical mechanisms may be involved in triggering the adverse reactions that lead to shared common clinical signs such those observed for NMS or NMS-like symptoms.

Although not confirmed by *in vivo* PET studies, reduced *DRD2* transcription levels have been reported by *in vitro* assay for the G-allele of this variant. Moreover, it is generally accepted that a blockade of dopamine receptor D_2 is one of the main mechanisms underlying NMS onset.

Thus, analysis of transcription factor (TFs) consensus sites by PROMO and AliBaba2.1 bioinformatic tools revealed changes of the consensus binding site of TFs resulting from the presence of the G allele versus the GG allele. In particular, the consensus binding sites for the Signal transducer and activator of transcription 4 (Stat-4) and the Transcriptional repressor protein YY1 (YY-1) were lacking in presence of G allele. These TFs are expressed in several district of CNS including regions involved in the regulation of body temperature, raising the hypothesis that alteration of the D_2 density in some neuronal population may confer higher susceptibility to adverse reactions to antipsychotics. Further studies are, however, needed to understand the role of this variant in regulating *DRD2* and in NMS onset.

In conclusion, rs1799732 may be a promising pharmacogenetic biomarker associated with a greater risk of developing NMS upon antipsychotic therapy. This finding may be prognostic to evaluate the risk of the therapeutic plans associated to first- or second- generation antipsychotics and it may be useful in the clinical practice to personalize the therapy minimizing the risk of adverse reactions.

ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
6-APB	6-(2-aminopropyl)benzofuran
AADC	Aromatic L-amino acid decarboxylase
ADHD	Attention-deficit hyperactivity disorder
ADR	Adverse drug reaction
ADRH	Antipsychotic drug-related heatstroke
ANKK1	Ankyrin repeat and kinase domain containing 1 gene
AP2-alpha	Transcription factor AP-2-alpha
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BDZs	Benzodiazepines
CACNA1S	Calcium voltage-gated channel subunit alpha1 S gene
CCD	Central Core Disease
C-ets-1	Protein C-ets-1
CNIT	National Centre for Toxicological Information
CNS	Central Nervous System
CHCT	Caffeine halothane contracture test
CHS	Classical heatstroke
COMT	Cathecol-O-methyltransferase
COX2	Cyclo-oxygenase type 2
CPK	Creatine phosphokinase
CSF	Cerebrospinal fluid
CTZ	Chemoreceptor trigger zone
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
DHPR	Dihydropyridine receptor
DIC	Disseminated intravascular coagulation
DOPAC	3,4-dyhydroxyphenylacetic acid

DRD2	Dopamine receptor D_2 gene
DRD3	Dopamine receptor D_3 gene
DMD	Duchenne Muscular Dystrophy
DSM-III	Diagnostic and Statistical Manual of Mental Disorders, Third Edition
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
ECT	Electroconvulsive Therapy
EHS	Exertional heatstroke
EM	Extensive metaboliser
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EMHG	European Malignant Hyperthermia Group
EPI	Epinephrine
EPR-3	PGE2 receptor 3
EPS	Extrapyramidal Symptoms
FGA	First generation antipsychotic
Foxp3	Forkhead box protein P3
GI	Gastrointestinal
gDNA	Genomic DNA
HIV	Human Immunodeficiency Virus
HS	Heatstroke
HTR1A	5-hydroxytryptamine receptor 1A gene
HTR2A	5-hydroxytryptamine receptor 1A gene
HVA	Homovanillic acid
IBMZ	[^{123}I] Iodobenzamide
IEC	International expert consensus
IL-1	Interleukin-1
IL-6	Interleukin-6

IM	Intermediate metaboliser
IVCT	In vitro contracture test
LAT1	Large neutral amino acid transporter 1
LPS	Lipopolysaccharide
LSD	Lysergic acid diethylamide
MAF	Minor allele frequency
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-N-ethyl-amphetamine
MDMA	3,4-methylenedyoximethamphetamine (ecstasy)
MH	Malignant Hyperthermia
MH+	MH susceptible
MH-	MH no susceptible
MHE	MH equivocal
MHN	MH normal
MHS	MH susceptible
MMD	Multi-minicore Disease
NAMHG	North American Malignant Hyperthermia Group
NE	Norepinephrine
NFE	Non-Finnish European population
NMS	Neuroleptic Malignant Syndrome
NPSs	Novel psychoactive substances
NSAIDs	Nonsteroidal anti-inflammatory drugs
PCC	Poison Control Center
PET	Positron emission tomography
PGE_2	Prostaglandin E2
PHEO	Phaeochromocytomas
PM	Poor metaboliser
PMMA	Paramethoxymethamphetamine
PNS	Peripheral nervous system
PO/AH	Preoptic and anterior hypothalamus
PTSD	Post-traumatic stress disorder

rCBF	Regional cerebral blood flow
$rCMRO_2$	Regional cerebral metabolic rate of oxygen
RYR1	Ryanodine receptor 1 gene
RyR1	Ryanodine receptor 1
SGA	Second generation antipsychotic
SNRI	Serotonin and norepinephrine re-uptake inhibitor
SNS	Sympathetic nervous system
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
SPECT	Single-photon emission computed tomography
SR	Sarcoplasmic reticulum
SRI	Serotonin reuptake inhibitor
SS	Serotonin Syndrome
SSRI	Selective serotonin reuptake inhibitor
STAT4	Signal transducer and activator of transcription 4 gene
Stat-4	Signal transducer and activator of transcription 4
T_3	Triiodothyronine
T_4	Thyroxine
TCA	Tricyclic antidepressants
TF	Transcription factor
TFII-I	General transcription factor II-I
TH	Tyrosine hydroxylase
TNF-α	Tumour necrosis factor alpha
TPH	Tryptophan hydroxylase
T-tubule	Transverse tubule
TSS	Transcription start site
UM	Ultrarapid metaboliser
VMAT2	Vesicular monoamine transporter 2
WBC	White blood cells
YY1	YY1 transcription factor gene
YY-1	Transcriptional repressor protein

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1 INTRODUCTION

Human normal body temperature is around 37°C and keeping it within narrow limits is critical to survival (Tansey and Johnson, 2015). This is achieved by a homeostatic thermoregulation system coordinated by the hypothalamus that maintains a dynamic balance between heat production and heat loss, so that core body temperature remains constant (Boulant, 2000; Tansey and Johnson, 2015).

Body thermoregulation system can be dysregulated by several different xenobiotics, including both medications and substances of abuse (Rusyniak and Sprague, 2005). The resultant hyperthermia is unresponsive to antipyretics and often results in life-threatening complications such as an intense skeletal muscle hypermetabolic reaction that leads to muscle rigidity, rhabdomyolysis, secondary hyperkalaemia, metabolic acidosis, disseminated intravascular coagulation, and multi-organ failure (Rusyniak and Sprague, 2005; Paden MS, Franjic L, 2013).

The main conditions associated to drug-induced hyperthermia and muscle rigidity are Malignant Hyperthermia (MH), Neuroleptic Malignant Syndrome (NMS), and Serotonin Syndrome (SS) that are triggered by different kinds of drugs, such as halogenated anaesthetic gasses and succinylcholine, antipsychotics, and serotonergic drugs or substances of abuse, respectively (Paden MS, Franjic L, 2013).

Patients presenting a suspected drug-induced syndrome should be admitted to hospital for close monitoring and treatment. In all of this cases, a revision of patient's history, a careful evaluation of any drugs taken and of the clinical picture can help in diagnosis (Jamshidi and Dawson, 2019). However, manifestations may be very similar making differentiation of these conditions one from the other difficult, especially as regards NMS and SS (Strawn, Keck and Caroff, 2007; Tse *et al.*, 2015; Pileggi and Cook, 2016; Jamshidi and Dawson, 2019; Simon and Keenaghan, 2020).

MH (OMIM: 145600) is a pharmacogenetic disorder of the skeletal muscle triggered by exposure to halogenated anaesthetic gasses or muscle

relaxant succinylcholine resulting in a dysregulated calcium release from sarcoplasmic reticulum with consequent development of a hypermetabolic state, in genetically predisposed patients (Rosenberg *et al.*, 2015; Watt and McAllister, 2020). Activation of the sympathetic nervous system and catecholamines release are likely to occur consequently to the hypermetabolic state, but dysregulation of calcium release in skeletal muscle cells due to exposure to triggering agents remains central for MH development (Rusyniak and Sprague, 2005).

Although their clinical presentation may be similar to MH, NMS and SS are caused by centrally active drugs with dopamine antagonist and serotonin agonist properties, respectively (Nisijima, 2015). The increasing diffusion of the use of second-generation antipsychotics and antidepressants with serotonergic action makes it difficult to distinguish between NMS and SS in patients presenting with hyperthermia, muscle rigidity, sympathetic nervous system lability, and altered mental status following exposure to both kinds of medication (Rusyniak and Sprague, 2005). Furthermore, clinical manifestation presenting with SS and NMS overlapping feature have been described also in association with certain substances of abuse, such as MDMA and cocaine (Daras *et al.*, 1995; Demirkiran, Jankovic and Dean, 1996; Wetli, Mash and Karch, 1996; Russell *et al.*, 2012).

Given similarities in the clinical presentation, common mechanism and genetic predisposition have been proposed, but remain so far unclear (Daras *et al.*, 1995; Demirkiran, Jankovic and Dean, 1996; Gurrera, 2002; Kawanishi, 2003; Rusyniak and Sprague, 2005; Steele, Keltner and McGuiness, 2011; Ortiz *et al.*, 2020).

1.1 Mechanisms of thermoregulation

Human normal body temperature is around 37°C and keeping it within narrow limits is critical to survival, since life-threatening events such as protein denaturation or reduced membrane fluidity and enzymatic activity occur upon an excessive increase or decrease in body temperature, respectively (Tansey and Johnson, 2015; Morrison, 2016). This is achieved by a homeostatic thermoregulation system coordinated by the hypothalamus that maintains a dynamic balance between the heat produced by metabolism and the heat lost, so that body temperature remains constant (Boulant, 2000; Tansey and Johnson, 2015).

When talking about body temperature, usually both central core and peripheral shell temperature are considered. The core temperature is the temperature within the internal organs, while the shell temperature refers to the skin (Tansey and Johnson, 2015). The hypothalamus, especially the preoptic anterior hypothalamus, coordinates and integrates inputs coming from peripheral thermoreceptors located in the skin and central thermoreceptors located in the hypothalamus, spinal cord, viscera and great veins (Boulant, 2000; Tansey and Johnson, 2015). In both districts two types of thermal receptors coexist: those sensitive to cold and those sensitive to warmth (Tansey and Johnson, 2015). The central control of thermoregulatory responses involves the existence of a hypothalamic "set-point", that under normal conditions is equal to 37 °C: signals coming from the peripheral and central thermal receptors are compared to the “set-point” and, if a discrepancy is detected, mechanisms for heat conservation or dispersion are activated (**Figure 1**) (Zocchi, 2012).

Activation of warmth sensitive receptors is processed by the pre-optic and anterior hypothalamus (PO/AH) that induces effector organ responses for heat dissipation, including skin blood vessels, sweat glands, endocrine tissue and level of basal vasoconstriction, mediated by noradrenergic innervation, is maintained in the blood vessels supplying both the glabrous and the non-glabrous skin (Tansey and Johnson, 2015).

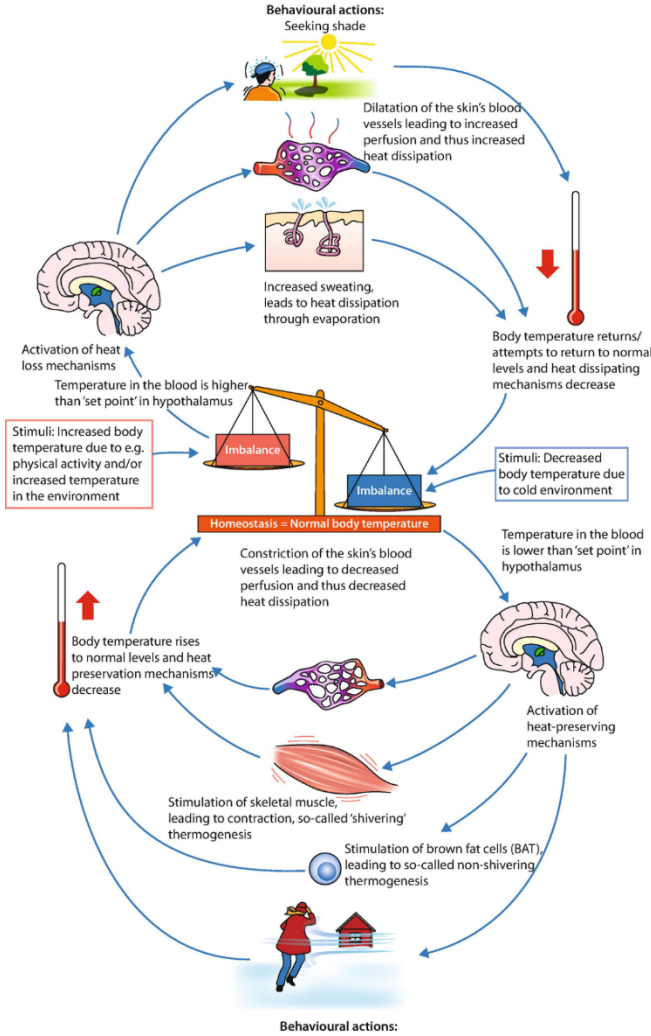

Figure 1. Mechanisms of thermoregulation in humans. In normal conditions, human body temperature is maintained around 37°C by a homeostatic control system regulated by the hypothalamus. Signals coming from the peripheral and central thermal receptors are compared to the "set-point" temperature in the hypothalamus and, if a discrepancy is detected, mechanisms for heat conservation or dispersion are activated. Image obtained from Grodzinsky and Sund Levander, 2020.

In response to an increase in temperature, skin blood flow is modified through vasodilation of skin blood vessels mediated by autonomic nervous system. In hairless skin, this is achieved passively by interrupting the noradrenergic activity which maintains the basal level of vasoconstriction, while in non-hairless skin an active mechanism of vasodilation mediated primarily by the release of acetylcholine can also occur (Charkoudian, 2003; Tansey and Johnson, 2015). Acetylcholine also mediates another important heat loss mechanism for humans. Indeed, the release of acetylcholine from sympathetic nervous fibres and its binding to muscarinic receptors on the sweat gland induce sweating, whose evaporation plays a major role in heat transferring from body to environment, especially when ambient temperature overcomes body temperature and during physical exercise (Tansey and Johnson, 2015; Baker, 2019). However, autonomic responses have a finite capacity to dissipate heat. Thus, behavioural adaptation plays an important role in preventing hyperthermia: humans can consciously adopt behaviours aimed at improving the exchange of heat with the external environment, such as reducing activity, seeking shelter in the shade, consuming cool drinks, anorexia and so on (Flouris, 2011; Tansey and Johnson, 2015).

Upon cold stimulus, effector organs responses are activated by the PO/AH to reduce heat loss, favouring the conservation of the endogenous heat produced by metabolism, and to increase its production, by increasing metabolic rate and voluntary or non-voluntary muscle activity (Zocchi, 2012). Vasoconstriction mediated by activation of α-noradrenergic receptor located within the blood vessels smooth muscle reduces blood flow to the skin surface (Tansey and Johnson, 2015). Furthermore, anterior hypothalamus induces the release of the thyrotropic hormone by the pituitary gland and the activity of sympathetic nerve fibres that innervate the adrenal medulla. The thyrotropic hormone stimulates the production of the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) which increase the metabolic activity, and therefore the heat production, by all of the body tissues (Zocchi, 2012). The catecholamines (adrenaline and noradrenaline) released by the adrenal medulla induce the process of non-shivering thermogenesis in the brown

adipose tissue (BAT), where the oxidative metabolism of fatty acids released by lipolysis is decoupled from the synthesis of adenosine triphosphate (ATP), producing heat (Zocchi, 2012; Tansey and Johnson, 2015). Non-shivering thermogenesis can be activated also by factors not related to temperature, such as infection processes (Tansey and Johnson, 2015). Changes in behaviour, such as increasing food intake, assuming a snuggled up body position, and putting on clothing are also considered responses to cold (Flouris, 2011; Tansey and Johnson, 2015). When these responses are not sufficient to keep an adequate body temperature, shivering thermogenesis occurs. Induced by the pre-optic anterior hypothalamus and mediated by the somatic motor cortex, shivering consists in an involuntary contraction of skeletal muscles during which ATP is consumed producing heat, instead of movement (Zocchi, 2012; Tansey and Johnson, 2015).

1.1.1 Fever and hyperthermia

The activity of the thermosensitive neurons located within the PO/AH can be influenced by some substances capable of inducing fever, that is an increase in the hypothalamic set-point obtained mainly by disinhibiting thermogenesis (Bartfai and Conti, 2010). These substances are collectively named *pyrogens* and can be distinguished in exogenous and endogenous. The lipopolysaccharide components of the bacterial cell wall (LPS), and other proteins or protein fragments that are released following bacterial or viral infections, inflammatory processes, tumours, trauma, and other tissue damages are examples of *exogenous pyrogens*. Exogenous pyrogens stimulate macrophages and other immune competent cells to synthetise cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor alpha (TNF-α), that are collectively named *endogenous pyrogens*. Endogenous pyrogens act on the hypothalamus increasing the set-point temperature, an effect mediated by prostaglandin E_2 (PGE_2) that modulates the set-point temperature by binding to PGE_2 receptor 3 (EPR-3) (Dinarello, 2004; Bartfai and Conti, 2010). The synthesis of PGE_2 takes place starting from arachidonic acid by various reactions catalysed by enzymes, among which cyclooxygenases, and especially type 2 cyclo-oxygenase (COX2), are of particular importance, as they are the target of nonsteroidal anti-

inflammatory drugs (NSAIDs) commonly used to treat fever and pain, such as acetylsalicylic acid, ibuprofen, and paracetamol (**Figure 2**) (Dinarello, 2004; Bartfai and Conti, 2010; Bacchi *et al.*, 2012). Thus, fever results from the coordinated responses of the effector organs activated by the hypothalamus to increase the body temperature; it responds to drugs inhibiting the synthesis of prostaglandins, such as acetylsalicylic acid, ibuprofen, and paracetamol but not to external cooling alone, as the body tends to maintain the temperature indicated by the hypothalamic set-point; moreover in rare instances it exceed 41°C (Mann *et al.*, 2003; Niven and Laupland, 2016).

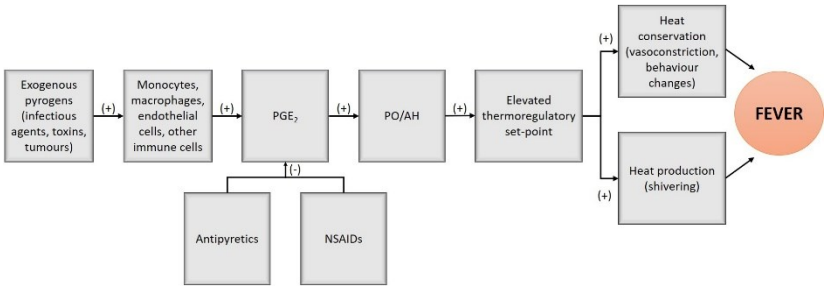

Figure 2. Mechanisms of fever. Fever results from the coordinated responses of the effector organs activated by the hypothalamus to rise body temperature upon stimulation by pyrogens. Adapetd from Dalal and Zhukovsky, 2006.

Abbreviations: PGE2, prostaglandin E_2; PO/AH, NSAIDs, nonsteroidal anti-inflammatory drugs; pre-optic and anterior hypothalamus; (+), activation; (-), inhibition.

Unlike fever, hyperthermia results from an altered central thermoregulation that is no longer able to coordinate the mechanisms of heat production and dissipation to maintain a thermal balance of the body (Zocchi, 2012). Acetylsalicylic acid, ibuprofen, paracetamol and other NSAIDs have no role in treating hyperthermia, since pyrogens, cytokines and prostaglandins are not involved (Niven and Laupland, 2016). Contrary, immediate cooling by physical methods is fundamental to reduce body temperature, that can commonly exceed 41°C, decreasing the occurrence of complications and improving survival (Niven and Laupland, 2016; Walter *et al.*, 2016). Hyperthermia can result from several causes, including high environmental

temperature, endocrine disorders, and drug-induced hyperthermia (Niven and Laupland, 2016).

Heatstroke (HS) is a potentially fatal medical condition characterized by body temperature elevation above 40°C, altered mental status, and multi-organ injuries resulting from exposure to hot and humid environment and/or intense physical activity. It can be distinguished in two forms: classical heatstroke (CHS) affecting mainly infants, pregnant women and elderly or debilitated individuals during heat waves, when elevated environmental temperature and humidity alter the body ability to dissipate heat; and exertional heatstroke (EHS) that is common among people practicing intense physical activity during summer, although hot and humid ambient temperature is a recurrent but not necessary condition for this form of heatstroke to develop (Liu *et al.*, 2020).

Endocrine disorders can cause hyperthermia. Indeed, thyrotoxicosis, phaeochromocytoma, and adrenal insufficiency can cause a raised body temperature that exceed the ability of the thermoregulatory mechanisms to dissipate heat, without changing the hypothalamic set-point (Tenner and Halvorson, 2013; Niven and Laupland, 2016). Thyroid hormones T_3 and T_4 are involved in basal metabolism and heat production. Thus, if abnormal quantities of these hormones are released, a hypermetabolic state can occur causing hyperthermia. This is what happens in thyrotoxicosis, a condition of thyroid-hormones excess that can be due to various causes, including increased production of thyroid hormones by the thyroid gland dependent on not dependent on pituitary stimulation, inflammatory states of the thyroid gland, and assumption of thyroid hormone or iodine (Tenner and Halvorson, 2013). Phaeochromocytomas (PHEO) are tumours of the chromaffin cells secreting norepinephrine (about 80-85% of cases), epinephrine or, in rare and clinically silent instances, dopamine. Mechanisms by which this kind of tumour produce hyperthermia is not completely understood, although it is supposed to be related to the induction of hypermetabolic state together with reduced heat loss due to altered vasodilation. Moreover, cases of PHEO inducing fever by IL-6 secretion have been reported (Zapanti and Ilias, 2006; Tenner and Halvorson, 2013). Adrenal insufficiency is characterized by

decreased levels of cortisol, that can cause severe hypotension, hypoglycaemia and immunodepression. Thus, patients affected by adrenal insufficiency are more predisposed to develop fever due to infections. However, they can develop also hyperthermia responding only to adequate steroid treatment, although the mechanism by which adrenal insufficiency causes hyperthermia has not been clarified so far (Tenner and Halvorson, 2013).

In addition to environmental and endocrine causes, a wide variety of drugs may induce hyperthermia (Niven and Laupland, 2016). Indeed, neurotransmitters norepinephrine (NE), dopamine (DA), and serotonin (5-hydroxitryptamine, 5-HT) have all been proposed to play an important role in hypothalamic regulation of body thermal balance. Reports involving DA and NE intrahypothalamic injections in rats showed that there are receptors for both DA and NE in the PO/AH which mediates a decrease in body temperature in rats, a response that could be blocked by their respective antagonists (Cox and Lee, 1980). Moreover, rats pre-treated with dopamine antagonists pimozide and haloperidol showed a reduced capacity to activate heat-loss mechanisms in response to infrared-lamp exposure (Cox, Kerwin and Lee, 1978). Further electrophysiological experiments performed on single PO/AH neurons showed that NE and DA stimulate warm-sensitive neurons reducing body temperature (Scott and Boulant, 1984; Mallick, Jha and Islam, 2002). Experiments performed in goats and rats provided evidence for the involvement of 5-HT in the activation of heat-loss mechanisms. De Roij *et al*. reported that in goats intracerebroventricular injection of both DA and 5-HT led to a reduction in body temperature by activation of heat loss mechanisms, such as dilation of ear blood vessels. Moreover, the hypothermic response to dopamine could be reduced by the administration of both the serotonin antagonist methysergide and the dopamine antagonist haloperidol, whereas serotonin thermoregulatory response was blocked only by methysergide, suggesting that 5-HT release is secondary to dopaminergic stimulation (De Roij *et al.*, 1978). Similar observations were reported by Cox and colleagues who identified the link within DA and 5-HT mediating heat loss within the PO/AH in rats (Cox *et al.*, 1980). Later works provided

information about receptors involved in thermoregulation responses, showing that 5-HT_{1A} agonists produced a marked hypothermia in rats, whereas 5-HT_{2A} agonists produced hyperthermia (Gudelsky, Koenig and Meltzer, 1986; Cryan *et al.*, 2000). Furthermore, alteration in central DA transmission by D_1 or D_2 dopamine receptors antagonism reduces the heat-dissipation capacity in exercising rats (Balthazar *et al.*, 2010). Thus, drugs altering the concentration of these neurotransmitters may interfere with the thermoregulatory systems of the body and lead to hyperthermia, including halogenated anaesthetic gasses and succinylcholine, centrally-acting dopamine antagonists such as antipsychotics and certain antiemetics, and serotonergic agents comprising both, drugs commonly used in the clinical practice as antidepressants and certain drugs of abuse, such as MDMA and cocaine (Rusyniak and Sprague, 2005). Indeed, these agents are the pharmacological triggers of the three primarily drug-induced heat illness: malignant hyperthermia, neuroleptic malignant syndrome and serotonin syndrome, respectively (Paden MS, Franjic L, 2013).

1.2 Malignant Hyperthermia

Malignant Hyperthermia (OMIM: # 145600) is an autosomal dominantly inherited pharmacogenetic disorder of the skeletal muscle which results in a hypermetabolic state triggered by exposure to halogenated anaesthetic gasses (e.g. halothane, isoflurane, enflurane) or muscle relaxant succinylcholine (Rosenberg *et al.*, 2015; Watt and McAllister, 2020). In rare instances, MH has been also described in relation to stressors such as heat and intense physical exercise (Tobin *et al.*, 2001).

In most cases, earlier signs of MH are difficult intubation due masseter muscle spasm and increase in end-expired carbon dioxide following the administration of a trigger anaesthetic agent or succinylcholine (Hadad, Weinbroum and Ben-Abraham, 2003; Paden MS, Franjic L, 2013). The progression of the syndrome includes tachycardia, increased oxygen consumption, acidosis, generalized muscle rigidity, rhabdomyolysis, hyperkalaemia, and body temperature elevation (Hadad, Weinbroum and Ben-Abraham, 2003; Rosenberg *et al.*, 2015). Death occurs in a 1.4%-5% of cases resulting from heart dysrhythmias, multiorgan failure and disseminated intravascular coagulation (DIC) (Paden MS, Franjic L, 2013; Rosenberg *et al.*, 2015; Watt and McAllister, 2020).

MH is estimated to occur in 1 per 250,000 cases involving general anaesthesia, but early signs that may indicate the development of MH occur in 1 per 16,000 anaesthetic procedures (Ording, 1985; Halliday, 2003). MH affects all ethnic groups worldwide, however variations of the incidence rate are reported depending on geographical region (Rosenberg *et al.*, 2015; Lu, Rosenberg and Li, 2017; Watt and McAllister, 2020). Males are affected twice than women, probably due to muscular body build (Brady *et al.*, 2009; Butala and Brandom, 2017; Lu, Rosenberg and Li, 2017). Incidence is higher among young people, the mean age of the patients experiencing the syndrome of 18.3 years (Rosenberg *et al.*, 2015).

The patients at risk for developing MH following exposure to a trigger agent include family members of patients affected and those who suffer a myopathic disorder (Paden MS, Franjic L, 2013; Rosenberg *et al.*, 2015).

Indeed, mutations of the *RYR1* gene have been identified also in patients affected by other myopathies such Central Core Disease and Multi-minicore Disease (Lawal, Todd and Meilleur, 2018). Central Core disease (CCD, OMIM: # 117000) is an uncommon non-progressive myopathy with mainly autosomal dominant inheritance that usually presents during infancy with proximal muscle weakness, hypotonia, delayed motor development, and skeletal alterations, such as scoliosis (Jungbluth, 2007). The name Central Core Disease is due to the fact that histochemical staining of muscle biopsy samples of patients affected by CCD shows regions of pale staining resulting from the absence or depletion of mitochondria (Jungbluth, 2007; Lawal, Todd and Meilleur, 2018). Frequently, patients affected by CCD test positive for MH susceptibility when undergoing muscle *in vitro* contracture test, thus they are at risk for MH episodes during general anaesthesia (Jungbluth, 2007). Multi-minicore disease (MMD, OMIM: # 255320) is another myopathy that has been suggested to be associated to MH (Rosenberg *et al.*, 2015). It is a congenital myopathy with an autosomal recessive inheritance characterized by the presence of many small areas showing disorganized sarcomere and lack of mitochondria in most muscle fibres, known as "mini cores" (Lawal, Todd and Meilleur, 2018). MMD is a clinically heterogeneous condition whose general features include neonatal hypotonia, delayed motor system development, and general muscle weakness, including bulbar, extraocular, and respiratory muscles (Jungbluth, 2007; Rosenberg *et al.*, 2015; Lawal, Todd and Meilleur, 2018). Recessive variants in *RYR1* gene have been associated with MMD, thus there may be a sub-set of *RYR1* variants related to both MH and MMD (Rosenberg *et al.*, 2015; Lawal, Todd and Meilleur, 2018). MH episodes can also occur in people with King-Denborough syndrome, a rare condition characterized by dysmorphic features, myopathies, and susceptibility to malignant hyperthermia (Dowling *et al.*, 2011). King-Denborough syndrome has been described both in presence and in absence of *RYR1* mutations, suggesting that genetic heterogeneity may be a characteristic of the syndrome (D'Arcy *et al.*, 2008; Dowling *et al.*, 2011).

Although the incidence of MH is rare, the prevalence of genetic variants associated with MH susceptibility is thought to be as high as 1 per 3000

individuals, with a more recent estimate of 1 per 400 (Gonsalves *et al.*, 2013; Rosenberg *et al.*, 2015). The difference between reported clinical incidence and genetic prevalence can be explained thanks to the fact that exposure to a causative agent is needed to trigger the onset of the syndrome, however most people carrying a genetic mutation predisposing to the syndrome never undergo general anaesthesia (Yang *et al.*, 2020). Moreover, MH shows variable penetrance in humans, as not all of the susceptible individuals develop the reaction when exposed to triggering agents (Yang *et al.*, 2020).

In normal conditions, skeletal muscle contraction starts with a depolarizing stimulus spreading across the sarcoplasmic membrane into the transverse tubule (T-tubule) of a muscle cell that activates the voltage-gated Ca^{2+} channel named dihydropyridine receptor (DHPR). Activated DHPR physically interacts with the ryanodine receptor type 1 (RyR1), a Ca^{2+} channel located in the membrane of the sarcoplasmic reticulum (SR). When RyR1 is activated and opened, Ca^{2+} is released from the SR into the cytoplasm leading muscle contraction (Yang *et al.*, 2020). MH is a skeletal muscle pathology due to an excessive release of calcium from skeletal muscle SR mediated by RyR1 following exposure to triggering agents (Watt and McAllister, 2020). Because of calcium release from SR, muscle rigidity develops causing an increase of oxygen consumption and carbon dioxide production, and a rise in body temperature. When ATP is depleted, the membrane integrity of the

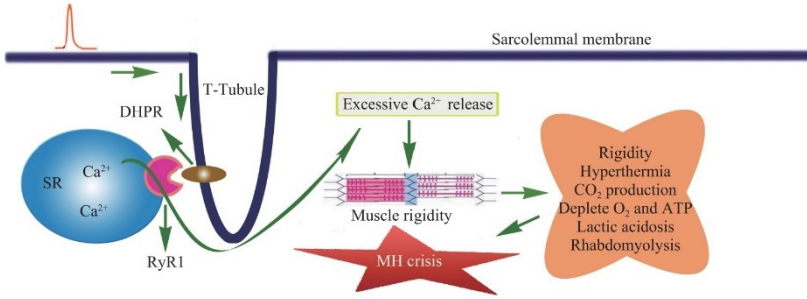

Figure 3. Proposed physiophathological mechanism for MH onset. Excessive Ca^{2+} release mediated by RyR1 induces muscle rigidity with increased consumption of O_2 and production of CO_2 and heat. Sustained muscle contraction also causes ATP consumption. When ATP is depleted, sarcolemmal membrane integrity is compromised and rhabdomyolysis occurs, so the cell content is released into the blood circulation. Adapted from Yang *et al.*, 2020.

skeletal muscle cells is compromised leading to rhabdomyolysis, so the cell content is released into the blood circulation (**Figure 3**) (Watt and McAllister, 2020; Yang *et al.*, 2020).

So far, two genes have been definitively associated with MH causative mutations: *RYR1* and *CACNA1S*. Human *RYR1* gene, located at Chromosome 19:38,433,691-38,587,564 forward strand (Ensemble Release 101, GRCh38.p13), encodes for RyR1 channel and is the major gene implicated in MH (Riazi, Kraeva and Hopkins, 2018; Watt and McAllister, 2020). More rarely, MH is related to a variant in the *CACNA1S* gene, that is located at human Chromosome 1:201,039,512-201,112,451 reverse strand and encodes the α-1S subunit of DHPR receptor (Monnier *et al.*, 1997; Stewart *et al.*, 2001). However, up to 50% of patients who experience MH do not carry any *RYR1* or *CACNA1S* variants, and the genetic basis of their MH susceptibility remains unclear (Riazi, Kraeva and Hopkins, 2018).

Muscle *in vitro* contracture test, performed by exposing biopsied leg muscle to halothane and caffeine with the aim to evaluate the different contractile response of normal and MH-susceptible muscle test, is the gold standard for the diagnosis of MH susceptibility (Rosenberg *et al.*, 2015). Two main protocols have been developed to perform the test: *in vitro* contracture test (IVCT) by the European Malignant Hyperthermia group (EMHG, https://www.emhg.org/) and caffeine halothane contracture test (CHCT) by the Malignant Hyperthermia Association of the United States (MHAUS, https://www.mhaus.org/) (European Malignant Hyperpyrexia Group, 1984; Larach, 1989; Fletcher, 1999; Rosenberg *et al.*, 2015). According to the EMHG protocol, a patient is considered susceptible to MH (MHS) when both caffeine and halothane test results are positive and not susceptible to MH (MHN) when both tests are negative. However, he or she can also be diagnosed as equivocal (MHE), but clinically considered MHS, when either a positive halothane or caffeine test alone is obtained (European Malignant Hyperpyrexia Group, 1984; Fletcher, 1999). In contrast, NAMHG protocol produces only two diagnosis: individuals are considered MH susceptible (MH+) when either a positive halothane or caffeine test alone is obtained, otherwise they are considered negative for MH (MH-) (Larach, 1989;

Fletcher, 1999). Overall, NAMHG and EMHG protocols show minor methodological differences, however the diagnoses produced by the two test are similar (Ørding *et al.*, 1997; Allen, Larach and Kunselman, 1998; Fletcher, 1999).

DNA testing requires only a blood sample to be performed, so it is becoming an interesting alternative to the invasive CHCT/IVCT test. However, because of genetic heterogeneity of MH susceptibility, muscle contracture tests remain the only reliable diagnostic tool for MH susceptibility diagnosis if genetic testing results negative (Rosenberg *et al.*, 2015; Riazi, Kraeva and Hopkins, 2018; Yang *et al.*, 2020).

As soon as MH is suspected, discontinuation of triggering agents and administration of dantrolene, that inhibits the calcium release in the skeletal muscle by binding to RyR1 protein, are paramount elements for the successful treatment of the syndrome (Rosenberg *et al.*, 2015; Watt and McAllister, 2020).

Several conditions presenting intraoperatively, such as sepsis, thyroid storm, and pheochromocytoma, may resemble MH. In these cases, end-expired carbon dioxide measurement helps in the differential diagnosis (Rosenberg *et al.*, 2015). When hyperthermia presents soon after anaesthesia, response to antipyretics is often useful in differentiating sepsis from MH (Rosenberg *et al.*, 2015). Young male patients with an occult dystrophinopathy, such as Duchenne Muscular Dystrophy (DMD, OMIM: # 310200), may experience a hyperkalemic cardiac arrest during or soon after exposure to succinylcholine or potent volatile anaesthetics (Larach *et al.*, 1997; Nathan *et al.*, 2005). Generally, these patients develop rhabdomyolysis but not hyperthermia and muscle rigidity, and dantrolene is ineffective. Moreover, dystrophinopathies come from mutations on X chromosome. Thus, these anaesthesia-related complications are not considered associated to MH (Rosenberg *et al.*, 2015). NMS is considered in the differential diagnosis of MH only in patients undergoing general anaesthesia, when both MH triggering agents and antipsychotics may be administered (Mann *et al.*, 2003). Although some symptoms appear to be similar, NMS does not occur

intraoperatively, because anaesthetics and muscle relaxants used during surgery probably inhibit the central mechanisms leading to its onset (Mann *et al.*, 2003). Both syndromes are successfully treated with dantrolene, however only NMS responds to dopamine agonists and electroconvulsive therapy (ECT) (Mann *et al.*, 2003; Krause *et al.*, 2004). Moreover, *in vitro* contracture studies performed on muscle biopsies coming from NMS patients have reported conflicting results (Adnet, Lestavel and Krivosic-Horber, 2000). Thus, NMS and MH are not considered to be associated (Keck, Caroff and McElroy, 1995; Rosenberg *et al.*, 2015).

Away from the surgical setting, MH-like symptoms may occur following the intrathecal injection of ionic contrast agents, and in serotonin syndrome (Rosenberg *et al.*, 2015). When ionic radiological contrast agents are injected intrathecally, a syndrome characterized by jerking involuntary muscle movements, seizures, and hyperthermia may occur. These manifestations may resemble MH, but the pathophysiology is different and depends on the contrast agent entering the cerebral ventricles. Treatment is symptomatic and includes cooling, administration of muscle relaxants, sedation, and mechanical ventilation (Rosenberg and Grant, 2004; Rosenberg *et al.*, 2015). Following ingestion of serotonergic agents, including certain drugs of abuse such as cocaine and MDMA, SS may develop. In the most serious forms, the syndrome may present with severe hyperthermia and muscle rigidity mimic MH; however, the presence of tremors, myoclonus, hyperreflexia, sweating, and hyperactive bowel sounds help in the differential diagnosis from MH (Scotton *et al.*, 2019). Underlying molecular mechanisms in common between MH and SS have been proposed, although with inconclusive and mixed results (Rusyniak and Sprague, 2005). Indeed, increased levels of serotonin have been detected in plasma of pigs during MH episodes and administration of serotonergic agents have shown to induce the development of hyperthermia and muscle rigidity in both MH-susceptible and MH-non susceptible pigs, but the administration of serotonin antagonists has not been shown to have a protective effect against the development of MH in susceptible pigs (Löscher *et al.*, 1990; Gerdes *et al.*, 1992; Löscher, Gerdes and Richter, 1994; Fiege *et al.*, 2003; Gerbershagen *et al.*, 2003).

1.3 Neuroleptic Malignant Syndrome

Neuroleptic Malignant Syndrome (NMS) is a rare but potentially fatal adverse drug reaction (ADR) associated with the intake of antipsychotic drugs and characterized by hyperthermia, muscle rigidity, altered consciousness and autonomic dysfunction (Caroff, 1980; Levenson, 1985; Caroff and Mann, 1993; Strawn, Keck and Caroff, 2007; Pileggi and Cook, 2016).

Recently, increasing awareness of NMS by physicians led to development of standardized diagnostic criteria and recommendations for proper medical treatment, with a direct impact on improving outcomes and declining morbidity and mortality (Oruch *et al.*, 2017; Velamoor, 2017). Despite these advances, there is a continuing need for study about NMS, since underlying pathophysiological mechanisms, genetic predisposition and relation with other drug-induced hyperthermic disorders remain open issues (Heyland and Sauve, 1991; Gurrera, 2002, 2011; Gillman, 2010a, 2010b; Margetić and Margetić, 2010; Duma and Fung, 2019; Jamshidi and Dawson, 2019; Simon, Hashmi and Callahan, 2020).

1.3.1 Historical background

In the early twentieth century, psychiatric medicine was mainly based on drastic somatic therapies: hydrotherapy, physical restrain, electroconvulsive therapy, sedation, lobotomy and sexual sterilization were practiced in the attempt to heal the mind by treating the body, but often without proven efficacy and causing severe adverse effects to patients (Braslow, 1997; Shorter, 1997).

It was in this framework that, at the beginning of 1950s, the introduction of chlorpromazine, the first antipsychotic, led to an important revolution in psychiatric practice (Shorter, 1997; Ramachandraiah, Subramaniam and Tancer, 2009; Carpenter and Davis, 2012). Chlorpromazine became available on prescription in France in 1952 and, within the next three years, its use in psychiatric practice spread around the world: the pharmacological revolution of psychiatry had started (Shorter, 1997; López-Muñoz *et al.*, 2004, 2005; Ban, 2007; Carpenter and Davis, 2012).

After the advent of chlorpromazine, the number of antipsychotics made available for clinical practice grew rapidly. However, chlorpromazine and other neuroleptic drugs soon demonstrated to have serious neurological adverse effects: extrapyramidal symptoms (EPS) including parkinsonism, dystonia, akathisia, acute and tardive dyskinesia started to be described in association with the administration of chlorpromazine, its derivates and even haloperidol, a butyrophenones synthesized in 1958 at a Belgian laboratory by Paul Janssen (Delay *et al.*, 1960; Shen, 1999; Granger and Albu, 2005; López-Muñoz *et al.*, 2005; Frankenburg and Baldessarini, 2008; Ramachandraiah, Subramaniam and Tancer, 2009).

Among the adverse effects of neuroleptic agents, also a rare but fatal disorder appeared: neuroleptic malignant syndrome was described for the first time in the French scientific literature in the 1960s by Delay and colleagues in association with haloperidol, however NMS was not definitely recognized in the English scientific literature until 1980, when Caroff's classic review was published and the description of the syndrome was included in the Diagnostic and Statistical Manual of Mental Disorders, Third Edition (DSM-III) published by the American Psychiatric Association (Delay *et al.*, 1960; Caroff, 1980; López-Muñoz *et al.*, 2005; Langan *et al.*, 2012; Velamoor, 2017).

1.3.2 Incidence

According to studies published between 1960 and 2003 reporting the occurrence of NMS among patients treated with antipsychotics, the incidence of NMS has varied widely, ranging from less than 0.07% to more than 3.23%, with an overall estimate of 0.99% (Delay *et al.*, 1960; Gelenberg *et al.*, 1988, 1989; Gurrera, Simpson and Tsuang, 2007).

Analysis performed at the same institution at different times have highlighted a declining trend in NMS incidence rates over time. Among the 1,162 patients who had been treated with antipsychotic drugs at a U.S. psychiatric hospital during 31-month period between 1984 and 1986, 13 patients had a diagnosis of NMS (1,10%) (Pope, Keck and McElroy, 1986). Later, the incidence of NMS reported at the same institution over a 47-month

period of study between 1986 and 1990 was about 7-fold lower (0.15%) (Keck, Jr and SL, 1991). Increased awareness of NMS early signs and symptoms by physicians with consequent prevention of its full-blown onset and safer prescribing practice may account for this reduction (Keck, Jr and SL, 1991; Buckley and Hutchinson, 1995; Buckley and Hasan, 1998; Adityanjee, Aderibigbe and Mathews, 1999). The perception that NMS incidence is diminishing has a direct impact on the detection of risk factors, effective prevention strategies and proper treatments (Gurrera, Simpson and Tsuang, 2007). However, some authors draw attention to the fact that study design limitations, diagnostic criteria adopted and the selection of the population at risk may have played an important role in the variation of NMS incidence rates (Adityanjee, Aderibigbe and Mathews, 1999; Mann *et al.*, 2003; Gurrera, Simpson and Tsuang, 2007).

Although this information is not suitable as a basis for determining the incidence of an adverse reaction as neither the total number of adverse reactions, nor the total number of patients exposed to the causative agent is known, Tse and his co-workers estimated that 404 new cases of NMS occurred in Canada between 1965 and 2012 by evaluating data coming from Canada Vigilance Adverse Reaction Online, a database that contains information about suspected adverse reactions of all drugs and medical devices approved in the Country (Tse *et al.*, 2015; Health Canada, 2020). Similarly, Trollor and colleagues searched the Australian Adverse Drug Reaction Advisory Committee database to analyse NMS cases reported in Australia between 1994 and 2010 identifying a total of 293 cases (Trollor *et al.*, 2012).

More recently, Schneider and colleagues estimated the incidence of NMS in Germany by analysing data coming from the database of the drug safety program “Arzneimittelsicherheit in der Psychiatrie” (AMSP) that assesses ADRs of psychiatric inpatients: between October 1993 and December 2015, 320,323 psychiatric inpatients treated with neuroleptics were monitored in the AMSP program and 52 cases of NMS were reported, with an incidence of 0.016% (Schneider *et al.*, 2020). These data appear to be consistent with a previous AMSP analysis that reported a 0.017% incidence for the period from

1993 to 2000 when among 86,439 patients exposed to neuroleptics, 15 developed NMS (Stübner *et al.*, 2004). Moreover, a study of comparable size performed in Denmark found 83 subjects diagnosed with NMS among 224,372 psychiatric inpatients and outpatients at risk, resulting in an incidence of 0.04% for the observation period from 1996 to 2007 (Nielsen *et al.*, 2012).

Therefore, NMS remains a rare adverse drug reaction that physicians should be aware of, as the number of cases is not negligible, in absolute terms, due to the increasing number of people treated with drugs that can cause the syndrome (Mann *et al.*, 2003; Verdoux, Tournier and Bégaud, 2010; Tse *et al.*, 2015).

1.3.3 The dopamine signalling pathway and the pathophysiology of NMS

Dopamine (DA) is synthesized starting from dietary phenylalanine that is converted to tyrosine by phenylalanine hydroxylase enzyme. Tyrosine enters the nerve terminal, where it is converted in L-dopa by the tyrosine hydroxylase, the rate limiting step in DA synthesis. Then, L-dopa is converted to DA by L-aromatic amino acid decarboxylase (AADC) and stored into secretory vesicles by the vesicular monoamine transporter 2 (VMAT2) until release (Rang *et al.*, 2016; Brunton, Hilal-Dandan and Knollmann, 2017).

Within the CNS, dopamine is an important neurotransmitter involved in the modulation of motor control, mood, behaviour, temperature and neuroendocrine function. Three are the main central dopaminergic pathways: nigrostriatal, mesocortico/mesolimbic, and tuberoinfundibular. The nigrostriatal pathway contains about 75% of the dopamine present in the brain and is made up of neurons present in the substantia nigra that project their axons into the dorsal striatum. Functionally, the nigrostriatal system is involved in motor control. Indeed, alterations in dopaminergic transmission in this pathway are related to Parkinson's disease (PD). Furthermore, D_2 receptor antagonism by antipsychotic drugs at the level of the nigrostriatal pathway can result in motor disorders. The cell bodies of neurons belonging to the mesocortico/mesolimbic pathway localize in the ventral tegmental area

of the midbrain, adjacent to the substantia nigra, and project their axons to the limbic system, especially *nucleus accumbens* and amygdala, and to the frontal cortex. The mesocortico/mesolimbic pathway is associated with emotions, reward, cognitive and behavioural functions. The euphoric effects of some drugs of abuse, such as amphetamines and cocaine, are related to the activation of this pathway. Moreover, dysfunctions in this pathway are related to drug addiction and schizophrenia. The tuberoinfundibular pathway comprises neurons originating in the hypothalamus that project to the pituitary, where dopamine is involved in regulating prolactin secretion and thus endocrine function (**Figure 4**) (Rang *et al.*, 2016; Brunton, Hilal-Dandan and Knollmann, 2017).

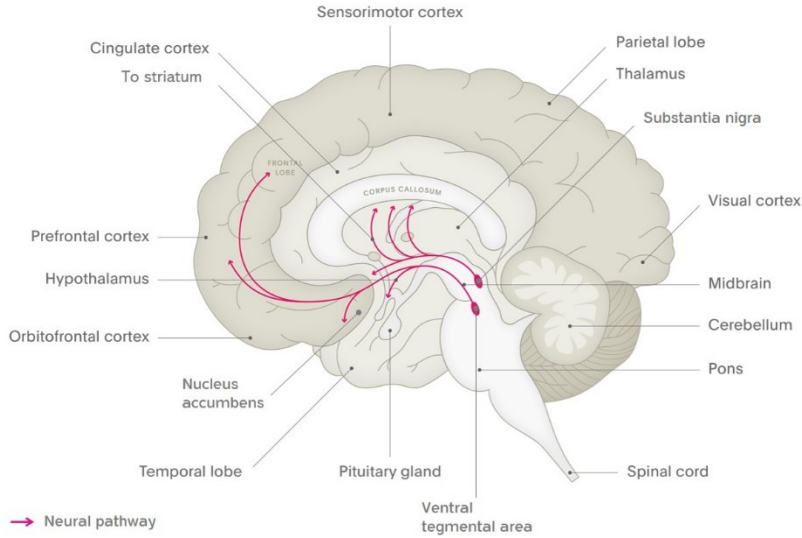

Figure 4. Brain dopamine pathways. Three are the main dopamine pathways in the brain: the nigrostriatal, mesocortico/mesolimbic, and hypothalamic - pituitary. The nigrostriatal pathway, projecting from *substantia nigra* to dorsal striatum, is involved in movement control. The mesocortico/mesolimbic pathway, that projects from the ventral tegmental area to *nucleus accumbens*, amygdala and frontal cortex, is involved in emotions and reward, cognitive functions, and social behaviour. The tuberoinfundibular pathway, projecting from hypothalamus to pituitary, modulates prolactin secretion. Adapted from Lundbeck Institute Campus (institute.progress.im).

Mediating these effects are the dopamine D_1 – like and D_2 – like receptors, that belong to the class of G protein-coupled receptors and are distinguished by their ligand specificities and their effects on G protein-mediated second-messenger systems. D_1 – like family comprises D_1 and D_5 receptors, whereas D_2 – like family comprises D_2, D_3 and D_4 receptors. Receptors belonging to D_1 – like family (D_1 and D_5) are coupled with G_s protein and activates adenylate cyclase (AC) to increase cyclic adenosine monophosphate (cAMP); whereas D_2- like receptors (D_2, D_3 and D_4) are coupled to G_i/G_o proteins which inhibit AC and reduce the synthesis of cAMP. Moreover, D_2 – like receptors can activate potassium channels and inhibit calcium channels. D_1 and D_2 receptors are abundantly expressed in the striatum, limbic system, and hypothalamus. In addition, receptor D_2 localizes also within the pituitary, where it is involved in the regulation of prolactin secretion. Indeed, dopamine release from hypothalamus reduced prolactin secretion by acting D_2 receptor within the pituitary. Moreover, D_2 receptor exists in two main isoforms that originate from alternative splicing: a protein of 443 aminoacidic residues that constitutes the D_2 long isoform, expressed mainly postsinaptically, and a protein of 414 aminoacidic residues that constitutes the isoform D_2 short, expressed mainly presynaptically, where it acts as somatodendritic autoreceptor to modulate and inhibit DA synthesis and release into the synaptic cleft. The D_2 receptor binds, with high affinity and specificity, many of the commonly prescribed antipsychotic drugs. Moreover, its density is altered in some neurological diseases. The D_3 receptor is less abundant than the D_2 receptor, and it is mainly expressed in the limbic system, in particular the *nucleus accumbens*, *substantia nigra* and the ventral tegmental area. The D_3 receptor is also thought to act as an autoreceptor, contributing to the regulation of dopamine release from the presynaptic terminal. Thus, D_1 and D_2 receptors are involved in motor control, emotions, reward mechanisms and cognition. Receptor D_3 also is thought to play a role in regulating these functions by exercising some minor modulatory effects on the activities of D_2 receptor. The D_4 receptor has a minor expression and it is located mainly in the limbic system and in the cortex. The D_5 receptor, which together with the D_1 receptor, belongs to the D_1-like family of receptors, is mainly located in the hippocampus, but is also found in the

substantia nigra, hypothalamus, striatum, cerebral cortex, *nucleus accumbens* and olfactory tubercule. From a functional point of view, the role of D_4 and D_5 receptors appears limited and regards mainly the modulation of some cognitive functions mediated by the hippocampus (Khan *et al.*, 1998; Missale *et al.*, 1998; Usiello *et al.*, 2000; Beaulieu and Gainetdinov, 2011; Rang *et al.*, 2016; Brunton, Hilal-Dandan and Knollmann, 2017). Although DA is present mainly in the brain, it has also some peripheral activities. DA circulating at low concentrations acts on vascular D_1 receptors inducing vasodilation. In contrast, at very high concentrations, it binds to vascular α-adrenoreceptors causing vasoconstriction. In the kidney, both D_1 and D_3 receptors are present with opposing effects on renin secretion. Finally, D_1 and D_2 receptors are both involved in the secretion of norepinephrine and epinephrine, with the stimulation of D_2 receptor inhibiting the release of NE from the sympathetic nerves and EPI from the adrenal medulla and the stimulation of D_1 inducing the release of catecholamines from the adrenal medulla (Beaulieu and Gainetdinov, 2011; Brunton, Hilal-Dandan and Knollmann, 2017).

Effects of DA are terminated by re-uptake into the presynaptic axon terminal via DA transporter (DAT) and metabolism mediated by the enzyme monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) whose main products are 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), excreted in the urine. Levels of DOPAC and HVA are considered indicators of DA turnover. DAT is targeted by several stimulant drugs, which obtain their euphoric effects by elevating DA levels within the synaptic cleft. Indeed, cocaine blocks DAT, resulting in a rise of synaptic DA. In contrast, amphetamine and related substances, such as methamphetamine, are taken up into the presynaptic neuron via the DAT. This also results in increased synaptic DA levels due to competitive re-uptake inhibition. Moreover, once inside the cytoplasm, these substances are able to enter secretory vesicles and cause an efflux of DA from the membrane by reversing the function of DAT, thus causing a further increase in synaptic DA (**Figure 5**) (Korpi *et al.*, 2015; Rang *et al.*, 2016; Brunton, Hilal-Dandan and Knollmann, 2017).

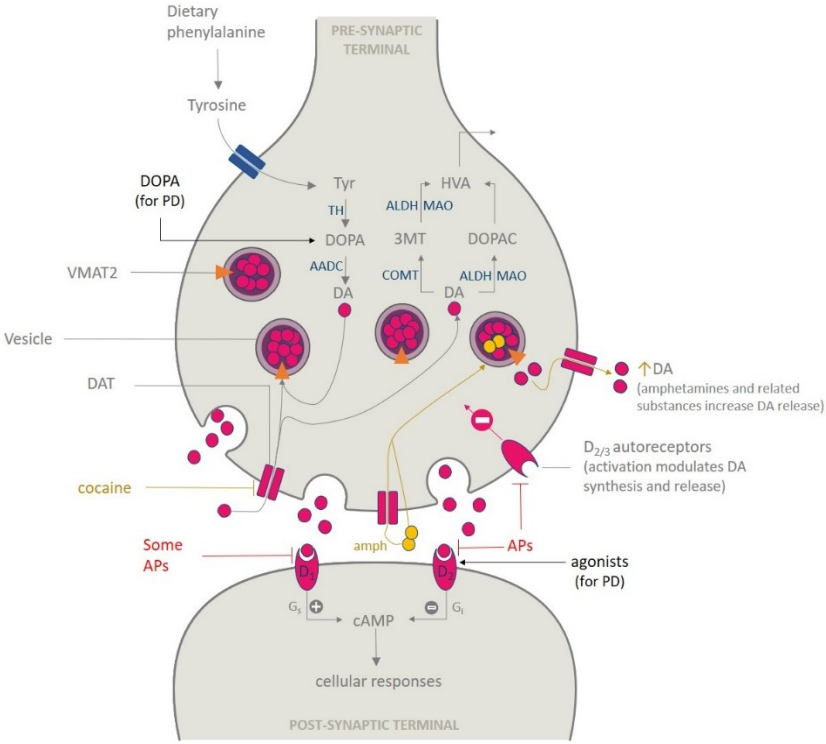

Figure 5. Schematic representation of a dopaminergic synapse. Drugs with an antagonist activity on dopamine D_2 receptor, including antipsychotics (in red) and certain antiemetics may cause NMS. Sudden withdrawal of DOPA or dopamine agonists used in management of Parkinson disease (in black) may cause events indistinguishable from NMS. The activity of stimulant drugs which obtain their euphoric effects by elevating DA levels within the synaptic cleft, is also shown (in yellow).

Abbreviations: 3-MT, 3-methoxytyramine; AADC, L-aromatic amino acid decarboxylase; ALDH, aldehyde dehydrogenase; amph, amphetamines; APs, Antipsychotics; cAMP, cyclic adenosine monophosphate; COMT, catechol- O-methyltranferase; D_1, dopamine type 1 receptor; D_2, dopamine type 2 receptor; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MAO, monoamine oxidase; PD, Parkinson disease; TH, tyrosine hydroxylase; Tyr, tyrosine; VMAT2, vesicular monoamine transporter 2.

Physiopathology underlying NMS onset are still unclear. However, central dopaminergic system is involved in regulation of temperature, motor coordination and muscle tone, mood and behaviour, thus it is generally accepted that central dopaminergic blockade, especially of dopamine receptor D_2, may be a critical step for the onset of NMS(Mann *et al.*, 2000; Strawn, Keck and Caroff, 2007; Berman, 2011; Velamoor, 2017).

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 in the brain may be a critical step for the onset of NMS. In a report published in 1995, Nisijima and Ishiguro measured the level of various metabolites in the CSF of 11 NMS patients and compared them with 8 age-matched normal controls. Compared with levels in normal subjects, HVA levels were significantly decreased during the active phase of NMS and after the recovery phase, supporting the central dopamine blockade theory of NMS (Nisijima and Ishiguro, 1995). Accordingly, the biochemical analysis performed by Kish *et al*. on the brains of three patients, two of which died of fatal catatonia and one died of NMS, detected about 50% – 70% reduction of levels of HVA in the striatum of one fatal catatonia patient and of the NMS patient (Kish *et al.*, 1990). Determination of blood flow and oxygen metabolism by Positron Emission Tomography (PET) in 3 treated patients with NMS showed an increase of regional blood flow (rCBF) and oxygen consumption ($rCMRO_2$) 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). Moreover, imaging of dopamine receptors occupancy performed with Single-Photon Emission-Computed Tomography (SPECT) using the radioactive ligand [^{123}I]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).

Accordingly, all antipsychotics causing NMS act as antagonists at dopamine receptors in the brain, and events indistinguishable from NMS can be occasionally caused also by the intake of other D_2 antagonists having no antipsychotic activity, such as certain antiemetics, or the sudden withdrawal of dopamine agonists in patients with no antipsychotic exposure (Spirt *et al.*, 1992; Caroff and Mann, 1993; Serrano-Dueñas, 2003; Wittmann *et al.*, 2016). Moreover, dopamine agonists play a role in NMS treatment (Pileggi and Cook, 2016).

Other neurotransmission pathways, including serotonin, acetylcholine, GABAergic and noradrenergic signalling, have been considered contributors to NMS. At this regard, Gurrera proposed that the clinical signs of autonomic dysfunction typically described in NMS patients, including tachycardia, tachypnoea, blood pressure lability, diaphoresis and incontinence, might be explained by hyperactivity and dysregulation of the sympatho-adrenergic system (Nisijima and Ishiguro, 1995; Gurrera, 1999; Mann *et al.*, 2000; Spivak *et al.*, 2000; Strawn, Keck and Caroff, 2007).

Given the similarities among NMS and MH clinical manifestations, a common pathogenetic mechanism involving a direct toxic effect on the peripheral skeletal muscles has been postulated. However, as regards NMS, *in vitro* contracture test studies and genetic investigations about the frequency of *RYR1* variants have provided mixed and inconclusive results so far (Keck, Caroff and McElroy, 1995; Miyatake *et al.*, 1996; Adnet, Lestavel and Krivosic-Horber, 2000; Sato *et al.*, 2010). As this regard, a further hypothesis has been proposed by Gurrera, who hypothesised that NMS might be interpreted as a "neurogenic form" of MH involving genetic mutations causing changes in calcium regulatory proteins within sympathetic neurons (Gurrera, 2002).

Whatever the trigger mechanism, the pathophysiology of NMS remains complex, since it involves as a first or secondary effect, many neurochemical and neuroendocrine systems (**Figure 6**) (Strawn, Keck and Caroff, 2007).

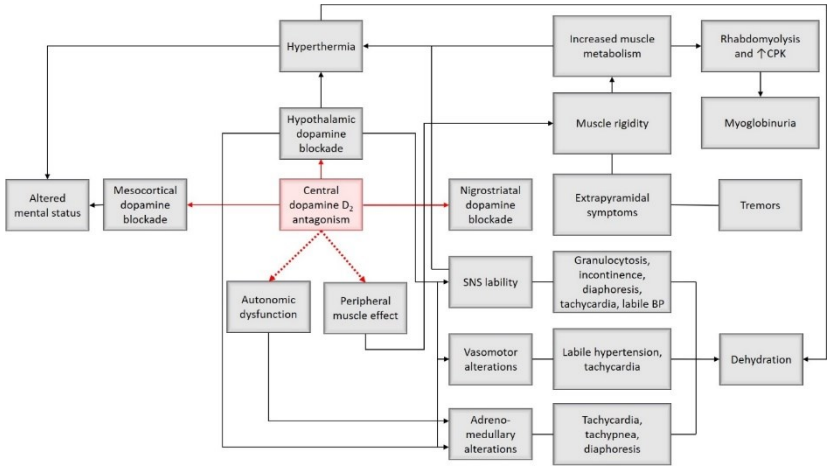

Figure 6. Pathophysiology of NMS. A schematic representation of the pathophysiologic mechanisms underlying NMS are shown, including elements of autonomic dysfunction and peripheral muscle effect. Adapted from Gurrera, 1999; Bhanushali and Tuite, 2004; Strawn, Keck and Caroff, 2007.

1.3.4 Causative agents

The onset of NMS is associated with the exposure to antipsychotic drugs (**Figure 7**) (Caroff, 1980; Levenson, 1985; Caroff and Mann, 1993; Strawn, Keck and Caroff, 2007; Pileggi and Cook, 2016). The term antipsychotic drugs - previously named neuroleptic drugs - conventionally refers to drugs commonly used in the management of schizophrenia, one of the most severe psychotic disorders (Rang et al., 2016). However, these drugs have also been approved for the treatment of other disturbances such as mania, agitation, bipolar disorder, Tourette syndrome, and hyperactivity (Sangani and Saadabadi, 2019). Moreover, they are used to treat attention-deficit hyperactivity disorder (ADHD), sexual behavioural disturbances, depression, severe anxiety, agitation and restlessness in the geriatric population, restlessness and pain in patients undergoing palliative care, insomnia, post-traumatic stress disorder (PTSD), disturbances related to substance abuse and dependence, and even nausea and vomiting (Rang et al., 2016; Sangani and Saadabadi, 2019).

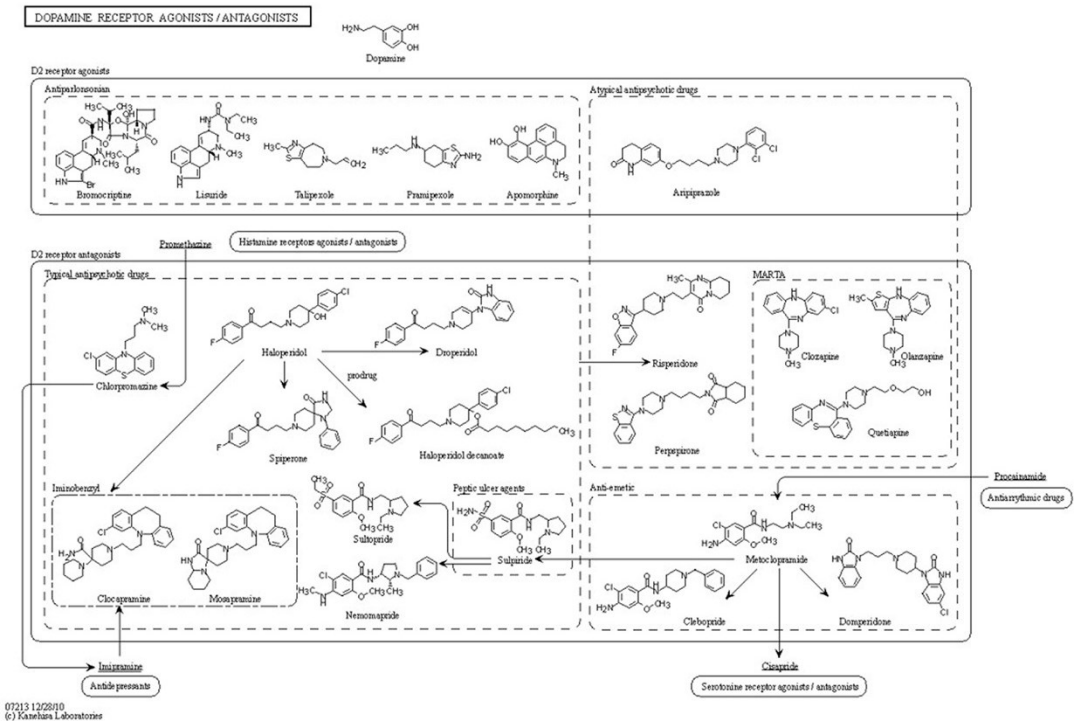

Figure 7. Dopamine D_2 receptor agonists and antagonists. Dopamine D_2 receptor agonists and antagonists, including certain NMS causative agents, are shown. Arrows indicate links among drug structures. Image adapted from: https://www.genome.jp/dbget-bin/www_bget?map07213).

Consistently with the growing off-label use of antipsychotics, NMS has started to be described as a syndrome affecting not only people with psychotic disorders, but also patients with other psychiatric or medical conditions (Tse et al., 2015).

The various symptoms of schizophrenia seem to be related to an altered function of the brain dopamine pathways (Rang *et al.*, 2016). In particular, the positive symptoms of schizophrenia, including delusions, allucinations, and disorganized thought, speech and behaviour, are thought to be caused by an excess of dopamine within the mesolimbic pathway. Whereas, the cognitive and negative symptoms of schizophenia, including dysfunction of communication, affect, socialisation, capacity for pleasure and motivation, are thought to be due to a deficit of dopamine activity in the mesocortical pathway (Toda and Abi-Dargham, 2007). Thus, one of the aims of treating schizophrenia is that to treat positive symptoms by decreasing dopamine activity in the mesolimbic pathway, and negative symptoms by increasing dopamine acrtivity in the mesocortical pathway, simultaneously (Rang *et al.*, 2016). Indeed, from a pharmacological point of view, antipsychotic drugs are characterized by an antagonistic activity on dopamine D_2 receptor, a factor to which the therapeutic effect is believed to be due (Rang *et al.*, 2016). However, based on the receptor profile, the incidence of extrapyramidal side effects, and the efficacy against negative symptoms, antipsychotic drugs can be divided into two groups: the so-called conventional, "typical," or first-generation antipsychotic agents (FGAs), and the "atypical" or second-generation antipsychotic agents (SGAs) (Rang *et al.*, 2016).

1.3.4.1 First-generation antipsychotics

The group of FGAs includes drugs that were developed first, such as chlorpromazine, a phenothiazine derivative produced in France in 1950, and haloperidol, a butyrophenone synthesized at the Belgian laboratory by Paul Janssen in 1958 (Granger and Albu, 2005; López-Muñoz *et al.*, 2005; Rang *et al.*, 2016). FGAs mechanism of action is based on antagonism of postsynaptic dopamine D_2 receptors. However, FGAs have effects on other receptors, such as histamine, muscarinic, and adrenergic, whose blockade is

related to side effects (sedation, anticholinergic properties, orthostatic hypotension). Moreover, typical antipsychotics D_2 receptors nonspecific binding throughout the brain is responsible for other undesirable adverse effects, such as worsen negative and cognitive symptoms due to D_2 receptor antagonism in the mesocortical pathway, prolactin levels increase resulting from D_2 receptor antagonism in the tuberoinfundibular pathway, and movement disturbances due to D_2 receptor antagonism in the nigrostriatal pathway (Rang *et al.*, 2016; Brunton, Hilal-Dandan and Knollmann, 2017; Ameer and Saadabadi, 2019).

1.3.4.1.1 Extrapyramidal movement disorders

Movement disorders are one of the major side effects of first-generation antipsychotic drugs (Rang *et al.*, 2016). They are often called extrapyramidal symptoms (EPS) as they involve the extrapyramidal system, that comprises the nigrostriatal pathway projecting from the *substantia nigra* to *corpus striatum* of the basal ganglia and plays an important role in motor function. The main EPS induced by antipsychotics are: dystonia, akathisia, parkinsonism, and tardive dyskinesia (Reeves and Swenson, 2008; Brunton, Hilal-Dandan and Knollmann, 2017; Darras and Volpe, 2018). Positron emission tomography (PET) studies suggested that 60–80% of D_2 occupancy is needed for FGAs to produce a therapeutic response, while the 75–80% of D_2 receptor occupancy is related to EPS onset (Kapur and Mamo, 2003). Thus, it is very difficult to avoid the overlap between the therapeutic D_2 receptor occupation and that causing adverse reactions, when using FGAs (Divac *et al.*, 2014).

Dystonia is an acute response to the sudden reduction of D_2 neurotransmission in the nigrostriatal pathway occurring from hours to days from the initiation of antipsychotic treatment and manifesting with muscle spasm of tongue, face, neck, and back (Brunton, Hilal-Dandan and Knollmann, 2017). The manifestations of dystonia often diminish over time and disappear following discontinuation of pharmacological treatment (Rang *et al.*, 2016).

It has been estimated that about 25% of patients treated with FGAs develop akathisia, a movement disorder characterized by a subjective and objective restlessness. Unresponsiveness to antiparkinsonian drugs, in favour of substances with significant cortical activity such as benzodiazepines and propranolol, seems to suggest that extra-striatal structures are involved in the onset of akathisia. However, this movement disorders remains poorly understood (Divac *et al.*, 2014; Brunton, Hilal-Dandan and Knollmann, 2017).

Motor disturbances resembling idiopathic Parkinson's disease may present within 5-30 days from the initiation of antipsychotic therapy with bradykinesia, rigidity and tremors responding to dose reduction, switching to a medication with less D_2 antagonism, and administration of antiparkinsonian drugs (Divac *et al.*, 2014; Brunton, Hilal-Dandan and Knollmann, 2017). Resembling a severe form of parkinsonism with extreme body rigidity, NMS can be considered also a rare but potentially fatal drug-induced movement disorder (Brunton, Hilal-Dandan and Knollmann, 2017; Duma and Fung, 2019).

Tardive dyskinesia can manifest with repetitive and stereotyped involuntary movements of the face, trunk, and extremities after months or years of treatment with antipsychotics, probably because of prolonged postsynaptic D_2 antagonism leading to an increase of the number of dopamine D_2 receptors in the *corpus striatum.* Tardive dyskinesia is considered a serious condition, as it is often irreversible, can worsen if antipsychotics are stopped and therapy is generally ineffective (Divac *et al.*, 2014; Rang *et al.*, 2016; Brunton, Hilal-Dandan and Knollmann, 2017).

1.3.4.1.2 Hyperprolactinemia

All FGAs have been associated with elevation in prolactin levels (Bostwick, Guthrie and Ellingrod, 2009). In physiological conditions, dopamine released by the neurons of the tuberoinfundibular pathway, which connects the hypothalamus with the pituitary gland, acts on D_2 receptor by inhibiting the secretion of prolactin (Rang *et al.*, 2016). Thus, antagonism of D_2 receptors by antipsychotic drugs can increase the plasma concentration of

prolactin, leading to both short- and long-term concerns. Short-term consequences of increased prolactin levels include gynecomastia and galactorrhoea affecting both men and women (Bostwick, Guthrie and Ellingrod, 2009; Rang *et al.*, 2016). Moreover, menstrual alterations and infertility can affect women, as well as sexual dysfunctions can occur in man. If hyperprolactinemia continues for a long time, decreased bone mineral density can occur in both men and women due to testosterone and estrogen deficiency, respectively (Bostwick, Guthrie and Ellingrod, 2009).

1.3.4.2 Second-generation antipsychotics

The introduction of clozapine to the US market in 1990 marked the beginning of the era of second generation antipsychotics (Shen, 1999). Generally, SGAs are potent antagonist at the 5-HT_{2A} receptor while showing lower affinity for D_2 receptor, especially in comparison to typical antipsychotic agents (Kapur and Mamo, 2003; Nasrallah, 2008; Brunton, Hilal-Dandan and Knollmann, 2017). Indeed, it has been observed that stimulation of 5-HT_{2A} receptors located on the axonal terminal of dopaminergic neurons by serotonin induces a reduction in dopamine release. Thus, by antagonizing 5-HT_{2A} receptors, SGAs prevented serotonin binding to 5-HT_{2A} receptors from reducing the release of dopamine (Steele, Keltner and McGuiness, 2011). Moreover, it has been proposed that SGAs bind loosely and fast dissociate from D_2 receptor, allowing for a normal dopamine neurotransmission (Kapur and Seeman, 2001). All this accounts for a reduced motor and endocrine side effects, and a better management of cognitive and negative symptoms compared to FGAs (Kapur and Seeman, 2001; Bostwick, Guthrie and Ellingrod, 2009; Divac *et al.*, 2014; Tuplin and Holahan, 2017).

More recently, agents with D_2 partial agonist properties, such as aripiprazole, have been developed. Partial agonists show an activity at D_2 receptors that is a fraction of the efficacy of DA (20%–25%), moreover they occupy the receptor antagonizing the binding of the endogenous agonist. Partial agonist antipsychotics require higher D_2 occupancy levels (80%–95%) than other antipsychotics (Brunton, Hilal-Dandan and Knollmann, 2017; Tuplin and Holahan, 2017). However, partial agonists activity at D_2 receptor

gives rise to a postsynaptic signal that is generally sufficient to avoid EPS onset, although cases have been reported (Desarkar, Thakur and Sinha, 2006; Henderson, Labbate and Worley, 2007; Chen and Liou, 2013; Tuplin and Holahan, 2017). As regards endocrine side-effects, partial agonism at D_2 receptor plays a role in reducing also the incidence of hyperprolactinemia, indeed aripiprazole administration does not result in significant alterations of prolactin levels (Rang *et al.*, 2016; Tuplin and Holahan, 2017).

Because SGAs cause fewer extrapyramidal symptoms than FGAs, they were thought to not cause NMS (Pileggi and Cook, 2016). However, many cases of NMS following exposure to second generation antipsychotic agents, often presenting without all the cardinal features or with a lower intensity of symptoms, have been reported. Some researchers have proposed the concept of atypical NMS to describe the form of NMS resulting more commonly from SGAs administration (Trollor *et al.*, 2012; Belvederi Murri *et al.*, 2015; Singhai, Kuppili and Nebhinani, 2019). However, an argument raised against this classification is that early diagnosis prevents the development of NMS in its classical form. Therefore, the form of NMS that develops following the administration of SGAs may not be atypical, but simply early diagnosed thanks to increasing awareness of the syndrome by clinicians (Pileggi and Cook, 2016; Oruch *et al.*, 2017; Velamoor, 2017).

1.3.4.3 Antiemetics

Cases of NMS have been reported also in association to certain antiemetics, such as metoclopramide and domperidone (Friedman, Weinrauch and D'Elia, 1987; Spirt *et al.*, 1992; Shaw and Matthews, 1995; Wittmann *et al.*, 2016).

Metoclopramide is a phenothiazine-like D_2 receptor antagonist that acts on the chemoreceptor trigger zone (CTZ), one of the main structures involved in emesis (Rang *et al.*, 2016; MacDougall and Sharma, 2020; Mirza and M Das, 2020). Moreover, metoclopramide acts as peripheral D_2 and 5-HT_3 receptors antagonist, and 5-HT_4 agonist. These properties do not increase the antiemetic effects of metoclopramide, but they are used in clinical practice to

increase motility of the gastrointestinal tract and accelerate gastric emptying (Rang *et al.*, 2016). Blockade of D_2 receptors in areas of the CNS other than CTZ can cause side effects such as EPS, hyperprolactinemia and even NMS (Friedman, Weinrauch and D'Elia, 1987; Shaw and Matthews, 1995; Rang *et al.*, 2016; Tufan *et al.*, 2016; Wittmann *et al.*, 2016).

Structurally associated to the butyrophenones, domperidone acts as antagonist of D_2 receptor in the CTZ, agonist of gastrointestinal 5-HT_4 receptor, and an antagonist of 5-HT_4 receptor in both the CTZ and the gastrointestinal tract. It is used mainly as antiemetic, but it also accelerates gastric emptying, and inhibits gastroesophageal reflux. Unlike metoclopramide, domperidone has a reduced ability to cross the blood-brain barrier and, therefore, it is less likely to cause central side effects (Reddymasu, Soykan and Mccallum, 2007; Rang *et al.*, 2016). However, cases of NMS following exposure to domperidone have been reported (Spirt *et al.*, 1992).

1.3.5 Risk factors

Although NMS occurrence is typically considered unpredictable, some risk factors that could favour the onset of the syndrome have been highlighted. These risk factors can be divided in four classes: pharmacological risk factors, environmental risk factors, demographic risk factors, and genetic predisposition (Tse *et al.*, 2015).

1.3.5.1 Pharmacological risk factors

Although a small number of subjects can exhibit symptoms also after having been in treatment for several months or years with the same antipsychotic agent, most of NMS cases occur within 30 days after initiating the antipsychotic therapy (Caroff and Mann, 1988; Berman, 2011; Velamoor, 2017). Variations of the treatment plan and in particular rapid escalation of the antipsychotic dose have been reported as factors predisposing to the onset of the syndrome (Langan *et al.*, 2012). In this regard, NMS has been described at all therapeutic doses and for all routes of administration. However, higher doses of antipsychotics and intramuscular or intravenous

routes of administration pose a greater risk of triggering NMS (Tse *et al.*, 2015).

Virtually all classes of drugs with D_2 receptor antagonist activity have been associated with NMS, including first- and second-generation antipsychotics and also certain antiemetics (Caroff and Mann, 1993). FGAs have generally been associated with a higher risk for NMS onset. However, current epidemiological data are not conclusive (Tse *et al.*, 2015). By evaluating data coming from a Canadian database that contains information about suspected adverse reactions of all drugs and medical devices approved in the Country, Tse and his co-workers estimated that 404 new cases of NMS occurred in Canada between 1965 and 2012, of which 342 cases were imputed to SGAs and 62 to FGAs. However, there is an overlap in these assessments due to the occurrence of antipsychotic polytherapy involving first- and second- generation antipsychotics simultaneously (Tse *et al.*, 2015). To avoid this confundent effect, Trollor and his co-workers searched data of NMS induced by antipsychotics monotherapy reported in the Australian Adverse Drug Reaction Advisory Committee database between 1994 and 2010, identifying 208 cases, of which 43 cases were imputed to FGAs and 165 to SGAs (Trollor *et al.*, 2012). Conversely, out of the 23 cases of NMS imputed to a single drug documented in the Germany drug safety program AMSP between October 1993 and December 2015, FGAs were implicated in the 60.9% of cases ($n = 14$) and SGAs in the remaining 39.1% ($n = 9$) (Schneider *et al.*, 2020). Similarly, a previous AMSP analysis reported 15 cases of NMS of which 11 involved a single drug, with a higher recurrence of FGAs than SGAs ($10\ vs\ 1$ cases, respectively) (Stübner *et al.*, 2004). What appears clearly is that SGAs are not free from the risk of triggering NMS, contrary to what was initially thought (Pileggi and Cook, 2016). Moreover, many author caution that cases of NMS following exposure to second generation antipsychotic agents may present without all the cardinal features of the syndrome or with a lower intensity of symptoms, making diagnosis more challenging (Belvederi Murri *et al.*, 2015; Uvais, 2018; Singhai, Kuppili and Nebhinani, 2019; Schneider *et al.*, 2020). Thus, awareness for NMS

should be maintained in individuals presenting with emerging signs and symptoms, regardless of antipsychotic class (Trollor *et al.*, 2012).

Finally, polypharmacy has been described as a risk factor for NMS, including both administration of more than one antipsychotic or administration of one or more antipsychotics and lithium (Buckley and Hutchinson, 1995; Nielsen *et al.*, 2012; Schneider *et al.*, 2020).

1.3.5.2 Demographic risk factors

Although NMS affects patients of all age groups, it has been reported more frequently among young and middle aged adults (Caroff and Mann, 1993; Gurrera, 2017).

Regardless of age, men have about 50% higher risk than women of being diagnosed with NMS (Gurrera, 2017). This appears to be mainly due to physical characteristics of the male gender that may trigger a more severe and recognizable presentation of the syndrome such as greater muscle mass that may produce a more severe muscle stiffness or sustain a greater metabolism leading to a more marked hyperthermia (Gurrera, 2017). Moreover, differences in exposure to antipsychotic drugs and in the doses used to treat men and women have been proposed to explain the higher frequency of NMS in males, rather than factors related to sex-linked transmission (Gurrera, 2017; Oruch *et al.*, 2017).

Factors associated to the patient's general health conditions, including psychiatric and medical concomitant pathologies can play an important role in influencing the risk of developing NMS (Caroff and Mann, 1993) .

1.3.5.3 Environmental risk factors

Extreme agitation, physical restraint, exhaustion, and dehydration have been reported to increase the risk of NMS development (Caroff and Mann, 1993; Viejo *et al.*, 2003). NMS seems to occur at any condition of environmental temperature and humidity (Caroff and Mann, 1993). However, elevated temperatures and humidity may facilitate the onset of the syndrome by interfering with body heat dissipation mechanisms (Tse *et al.*, 2015).

1.3.5.4 Genetic risk factors

Findings in pharmacogenetics indicate that the genetic polymorphisms are associated with the interindividual differences in drug responses concerning both efficacy and adverse reactions (Evans and McLeod, 2003).

Despite the rare frequency of the syndrome, NMS may occur in multiple members of a family, as described for twin brothers with schizophrenia, a mother and her two daughters, and two siblings with type II gangliosidosis, an hereditary metabolic disease (Deuschl *et al.*, 1987; Otani *et al.*, 1991; Manor *et al.*, 1997). Moreover, patients who experienced NMS remain at higher risk: rechallenge with antipsychotics is associated with a risk of developing NMS again as high as 30% (Caroff and Mann, 1988; Wells, Sommi and Crismon, 1988; Lazarus, Moore and Spinner, 1991; Tsutsumi *et al.*, 1998). This evidence support the hypothesis that genetic predisposition plays a role in the onset of NMS, although the presence of other concomitant factors is likely to be necessary since several reports show successful rechallenge after NMS resolution (Rosebush, Stewart and Gelenberg, 1989; Pope *et al.*, 1991; Lally *et al.*, 2019).

So far, a limited numbers of genetic variants have been studied in association with NMS susceptibility, although with inconclusive and mixed results (Kawanishi, 2003). Moreover, most of the studies are based on the Japanese population and only a few data coming mainly from case reports are available for other ethnic groups (Kawanishi, 2003; Del Tacca *et al.*, 2005; Živković *et al.*, 2010). Therefore, extensive analysis are required to identify genetic biomarkers of susceptibility to NMS (Kawanishi, 2003; Živković *et al.*, 2010).

1.3.5.4.1 Drug targets

Mechanisms underlying NMS onset are still unclear. However, it is generally accepted that central dopaminergic blockade, especially of dopamine receptor D_2, may be a critical step for the onset of NMS (Berman, 2011; Velamoor, 2017). Thus, many studies about genetic predisposition to NMS have focused on dopamine receptor D_2.

Ram *et al*., analysed the coding sequence of the D_2 receptor gene (*DRD2*) within a total of 12 patients with an history of NMS, including 10 Black and Caucasian unrelated individuals presenting sporadic NMS and the two Japanese sisters previously reported by Otani *et al.*, finding that one patient with sporadic NMS was heterozygous for the missense variant rs1800496, also named Pro310Ser (Otani *et al.*, 1991; Ram *et al.*, 1995). However, the finding of only one patient having an history of NMS who carried the variation did not achieve statistical significance (Ram *et al.*, 1995).

Historically located within *DRD2* gene, rs1800497 is a missense variant now mapped at position 11:113400106 into exon 8 of the adjacent *ANKK1* gene that encodes ankyrin repeat and protein kinase domain-containing protein 1 (Grandy *et al.*, 1989; Neville, Johnstone and Walton, 2004). However, it is still regarded as an affecting factor for *DRD2* gene regulation (Noble, Blum and Khalsa, 1991; Thompson *et al.*, 1997; Pohjalainen *et al.*, 1998; Jönsson *et al.*, 1999; Hirvonen, Laakso, *et al.*, 2009; Hirvonen, Lumme, *et al.*, 2009a; Savitz *et al.*, 2013). Two Japanese studies, performed respectively on a cohort of 15 and 17 NMS patients, 13 of which were in common, suggested a putative association between NMS susceptibility and the rs1800497 variant (Suzuki *et al.*, 2001; Mihara *et al.*, 2003). Subsequently, two additional studies performed on larger samples of patients (49 and 32 Japanese patients with NMS, respectively) did not observe any association of this variant with the NMS onset (Kishida *et al.*, 2003, 2004).

The variant rs1799732, traditionally named -141C ins/del, is located at chr11: 113475529 within the promoter region of *DRD2* gene (Arinami *et al.*, 1997). An altered receptor binding in the *striatum* has been observed in healthy subjects carrying this variant (Jönsson *et al.*, 1999). Moreover, the transactivation assay of a reporter gene under the control of *DRD2* promoter region showed a downregulation of its expression in presence of the of the -141C deletion (G allele) (Arinami *et al.*, 1997). The association between NMS susceptibility and this variant was investigated by two studies, producing conflicting results. Indeed, one study found no difference in the allelic and genotypic frequencies of variant between patients and controls, while the other one observed a significantly elevated frequency of the -141C deletion

(G allele) in NMS patients compared to controls (Mihara *et al.*, 2003; Kishida *et al.*, 2004). Also known as Ser311Cys, rs1801028 is a missense variant located within exon 7 of *DRD2* gene and it has been suggested to affect the interaction between D_2 receptor and its G protein (Cravchik, Sibley and Gejman, 1996). However, it resulted in a lack of association with NMS susceptibility (Kishida *et al.*, 2004).

Although the role of other dopamine receptors in the onset of NMS is undetermined, the missense variant rs6280 of the gene that encodes for dopamine D_3 receptor (*DRD3*) has also been investigated, but it resulted in an absence of association with NMS susceptibility (Kishida *et al.*, 2003; Mihara *et al.*, 2003).

Serotonin syndrome is the most common condition that can be misdiagnosed as NMS and sometimes the two syndromes can present with indistinguishable manifestations (Rusyniak and Sprague, 2005; Strawn, Keck and Caroff, 2007). Thus, some authors proposed that an overlap between their underlying mechanisms may exist (Steele, Keltner and McGuiness, 2011). Assuming a putative involvement of serotonin receptors in NMS onset, Kawanishi and colleagues examined the frequencies of three missense variants of the *HTR1A* (rs200894913) and *HTR2A* (rs1805055 and rs6314) genes, encoding respectively 5-hydroxytryptamine receptor 1A and 2A, in 29 Japanese NMS patients compared to controls. However, no allelic variant was found among NMS patients (Kawanishi, Hanihara, *et al.*, 1998).

Patients with NMS have some manifestations common to MH as well. Therefore, some investigations have been performed to assess the frequency of *RYR1* variants in patients with NMS. Miyatake and colleagues studied six previous MH-associated *RYR1* gene mutations in 10 Japanese patients with a history of NMS, finding no variants in any of them (Miyatake *et al.*, 1996). More recently, Sato and co-workers conducted post-mortem screening of the *RYR1* gene in 11 psychiatric patients who were suspected of having died of NMS and two of them were found to carry two different missense variants of this gene. In particular, one of these two variants had previously been reported in a Japanese individual with MH, while the other had never been reported in

Japanese individuals with MH, but it involves an amino acid residue highly conserved in various species that is located close to a variant which is known to be associated with MH. However, further studies are needed to establish a possible correlation between mutations in the *RYR1* gene and NMS (Sato *et al.*, 2010).

1.3.5.4.2 Drug metabolizing enzymes

Cytochrome P450 (CYP) 2D6 is the enzyme involved in drug metabolism most studied from a pharmacogenetic point of view, as it is responsible for the metabolism of approximately 25% of currently marketed drugs, including antipsychotics, antidepressants and antiemetics (Teh and Bertilsson, 2012). It is encoded by the *CYP2D6* gene (MIM: * 124030), located on chromosome 22 in correspondence with the chromosomal band q13.2 (Eichelbaum *et al.*, 1987). It is a highly polymorphic site, with more than 80 allelic variants described so far (see https://www.pharmvar.org/genes for updates), encoding for forms of the enzyme with enhanced, normal, reduced, or absent activity (Bertilsson *et al.*, 2002). The phenotypes resulting from the combination of these allelic variants can be divided according to the enzymatic activity into: extensive (EM), poor (PM), intermediate (IM), and ultrarapid (UM) metabolizers (Teh and Bertilsson, 2012). PMs lack the functional enzyme due to a deletion of the gene or SNPs that result in the expression of an inactive form of the enzyme. PMs show reduced drug metabolism and are therefore more prone to adverse reactions even at therapeutic doses. Conversely, UMs have more than two functional alleles due to duplication events. They exhibit increased drug metabolism and therefore may require higher doses to achieve therapeutic effect. Between these two extremes, there are the EMs that are homozygous or compound heterozygous carriers of normal or enhanced activity alleles, and IMs that are homozygous or compound heterozygous carriers of reduced functional alleles (Teh and Bertilsson, 2012). The *CYP2D6* gene shows a high variability between different populations, with a different frequency of alleles in ethnic groups and population-specific allelic variants (Bertilsson *et al.*, 2002). Regarding NMS, two Japanese patients homozygous for the *CYP2D6* * 10 allele encoding a reduced activity form of the enzyme were reported by Kawanishi and colleagues (Kawanishi,

Shimoda, *et al.*, 1998). However, a subsequent study comparing the frequency of this allele between NMS patients and controls yielded negative results (Kawanishi *et al.*, 2000). No association with NMS was found also for *CYP2D6* * 3 and * 4 alleles, that account for PM phenotype (Kawanishi *et al.*, 1997).

1.3.6 Clinical features

Clinical presentation and development of NMS can be variable, making diagnosis challenging, especially in the initial phase (Oruch *et al.*, 2017; Velamoor, 2017). However, the four main symptoms of NMS are generally recognized as hyperthermia, muscle rigidity, altered consciousness and autonomic dysfunction following exposure to a causative agent, usually a drug with dopamine-receptor antagonist activity (Caroff, 1980; Caroff and Mann, 1993; Berman, 2011; Velamoor, 2017).

Patients typically develop NMS within hours or days after exposure to the offending agent, with most of cases occurring within 30 days after initiating neuroleptic therapy, however a reduced number of subjects can exhibit symptoms also after having been in treatment for several months or years (Caroff and Mann, 1988; Berman, 2011; Velamoor, 2017). Pooled data coming from 153 clinical cases of NMS published between 1980 and 1990 suggest that the clinical course of NMS usually starts with changes in mental status, characterized by delirium or consciousness fluctuations from confusion to coma (Velamoor *et al.*, 1994; American Psychiatric Association, 2013). Generalized muscle rigidity, variable from a milder to a more severe form, described as “lead-pipe” and eventually associated with rhabdomyolysis, typically develops before body temperature elevation (Caroff, 1980; Caroff and Mann, 1993; Velamoor *et al.*, 1994; American Psychiatric Association, 2013). Tremors are commonly reported; other extrapyramidal signs, such as blefarospasm, oculogyric crisis, trismus, nystagmus, dystonia, dysphagia and dysarthria can also be present as a result of augmented muscular tone, but are less frequently reported (Caroff and Mann, 1993; American Psychiatric Association, 2013; Tse *et al.*, 2015). In the majority of cases, changes in mental status and muscle rigidity precede

hyperthermia and autonomic dysfunction (Velamoor *et al.*, 1994). Hyperthermia, associated with profuse sweating and unresponsive to antipyretics, is a distinguish characteristic of NMS, but it can be delayed for several hours after the appearance of the early symptoms (Berman, 2011; American Psychiatric Association, 2013; Tse *et al.*, 2015; Oruch *et al.*, 2017). Autonomic dysfunction presents as tachycardia, tachypnoea, blood pressure instability, sialorrhea, diaphoresis, skin pallor and urinary incontinence (Berman, 2011; American Psychiatric Association, 2013). Once started, NMS symptoms can evolve very rapidly reaching the peak of intensity in 24-72 hours (Caroff, 1980; Berman, 2011; Oruch *et al.*, 2017).

So far, no laboratory abnormality has been identified as a specific biomarker for NMS diagnosis, however several hematologic and biochemical changes have been associated with the syndrome: elevated creatine phosphokinase (CPK) levels due to rhabdomyolysis, leukocytosis, increased inflammatory markers (C-reactive protein, fibrinogen, erythrocyte sedimentation rate), hypoxia, metabolic acidosis and iron deficiency may be present in subjects affected by NMS (Caroff and Mann, 1988; Berman, 2011; American Psychiatric Association, 2013; Tse *et al.*, 2015; Oruch *et al.*, 2017).

Results from cerebrospinal fluid (CFS) analysis and neuroimaging studies are usually normal, whereas about 50% of patients show a slowing of electroencephalographic pattern (Caroff and Mann, 1988; Berman, 2011; American Psychiatric Association, 2013; Oruch *et al.*, 2017).

The clinical features reported above describe NMS in its classic full-blown form, however cases of atypical NMS, characterized by subthreshold presentation of symptoms, such as with less muscle rigidity and less severe hyperthermia, have been described in literature resulting more commonly from second generation neuroleptics administration (Singhai, Kuppili and Nebhinani, 2019).

Post-mortem examination in fatal cases have reported nonspecific and heterogeneous findings, depending on complications occurred as a result of NMS (Downey *et al.*, 1992; Lannas and Pachar, 1993; Naramoto *et al.*, 1993; Thompson *et al.*, 1997; Gambassi *et al.*, 2006; American Psychiatric

Association, 2013; Musshoff, Doberentz and Madea, 2013; Angélique *et al.*, 2018; Matsusue *et al.*, 2018).

1.3.7 Diagnostic criteria

Based on clinical and laboratory features described by NMS case reports, several sets of diagnostic criteria for NMS have been developed across years (**Table 1**).

In 1985, Levenson proposed the first set of diagnostic criteria for NMS. His criteria were based on the presence of all three major (fever, rigidity, elevated CPK) or two major and four minor (tachycardia, abnormal blood pressure, tachypnoea, altered consciousness, diaphoresis and leukocytosis) signs to make a highly probable diagnosis of NMS, if other similar conditions have been ruled out (Levenson, 1985). Levenson's set of criteria are very inclusive but less specific, because of the possibility to diagnose NMS in absence of hyperthermia or rigidity, considered by most experts as cardinal features of NMS (Mann *et al.*, 2003; Tse *et al.*, 2015).

Pope and colleagues' criteria suggested that NMS was diagnosable based on the simultaneous presence of hyperthermia (> 37.5°C) in the absence of other known aetiologies, severe extrapyramidal symptoms and autonomic dysfunction. Altered consciousness, leukocytosis or elevated CPK could be used to make a retrospective diagnosis of probable NMS if one of the above criteria was not documented (Pope, Keck and McElroy, 1986). Subsequently, the authors made these criteria more restrictive by raising the fever threshold to 38°C (Keck PE Jr, Sebastianelli J, Pope HG Jr, 1989; Adityanjee, Mathews and Aderibigbe, 1999).

Considering NMS a continuous spectrum of pathophysiologic reactions to neuroleptics more than a specific syndrome, Addonizio *et al*. proposed a list of 10 signs and symptoms to identify NMS: hyperthermia (> 37.5°C, in the absence of other aetiologies), EPS, tachycardia, tachypnoea, elevated blood pressure, diaphoresis, incontinence, leukocytosis, confusion and elevated CPK. The occurrence of 5 symptoms (including fever and EPS, whose presence was an essential requirement for the diagnosis) within the same 48-

hours period allowed the identification of an NMS episode (Addonizio, Susman and Roth, 1986). These criteria gave as importance to non-specific findings like leucocytosis and elevated CPK as to specific manifestations like autonomic dysfunctions, resulting in a very flexible but potentially over-inclusive diagnostic instrument (Adityanjee, Mathews and Aderibigbe, 1999).

So far, the criteria presented have not explicitly included neuroleptic administration as a requirement for NMS diagnosis. As essential parameters for NMS diagnosis, Caroff's group proposed the presence of both hyperthermia and muscle rigidity in association with the exposure to a neuroleptic agent. Moreover, these criteria emphasize that NMS is a diagnosis of exclusion and other neuropsychiatric, organic, or drug-induced pathologies that may underlie the clinical picture should be ruled out before diagnosing the syndrome (Lazarus, Mann and Caroff, 1989; Caroff *et al.*, 1991; Caroff and Mann, 1993).

A consensus among investigators that hyperthermia and muscle rigidity associated with the use of neuroleptic drugs as cardinal features to make a diagnosis of NMS was reached with the diagnostic criteria published first in DSM-IV and then replicated in DSM-IV-TR (American Psychiatric Association, 1994, 2000; Velamoor, 2017). The DSM-IV gives very broad criteria for diagnosis of NMS that are essentially a modified version of criteria given by Caroff's group, however these criteria have a limited use in research and clinical management of patients as neither the critical values, nor the relative importance of the single diagnostic criterion have been established (Adityanjee, Mathews and Aderibigbe, 1999; Velamoor, 2017).

In the attempt to minimize false-positive rate without excluding the impact of atypical forms of NMS and establish a common vocabulary for research communications, Aditiyanjee's group developed a more strict set of criteria that distinguish among four types of NMS (Adityanjee *et al.*, 1988; Adityanjee, Mathews and Aderibigbe, 1999):

1. ***Type I***. This type refers to classical NMS induced by exposure to FGAs or other kinds of dopamine antagonists, such as certain antiemetics. Other causative agents are excluded. Very strict

parameters, based on the previous version of NMS diagnostic criteria proposed by Adityanjee's group in 1988, and the adoption of rating scales to measure the severity of symptoms are suggested to diagnose the syndrome, so that the inclusion of false-positive cases is minimized.

2. ***Type II***. This type refers to atypical NMS following exposure to SGAs. This condition should not be considered a milder form of type I NMS because it may also have a severe course, but the presence of EPS is not required to make a diagnosis.

3. ***Type III***. Induced by exposure to FGAs or SGAs, this condition does not meet the criteria for either type I or II NMS, as the clinical picture is not complete. Otherwise, it is strongly suspected to be NMS, probably in an incipient or aborted form.

4. ***Type IV***. This type includes miscellaneous conditions that resemble typical NMS; however, their onset is not associated with neuroleptics exposure, resulting from the withdrawal of dopaminergic agents, exposure to psychostimulants or administration of dopamine depleters.

According to the authors, only type I and type II should be considered as definitive NMS, whereas type III and type IV represent, at best, probable NMS. However, the characterization of these latter conditions may be useful in clinical practice, where more flexibility is needed (Adityanjee, Mathews and Aderibigbe, 1999).

In 2011, an international panel of NMS experts converged to establish a set of clinical features that are most important in making a diagnosis of NMS, the relative importance of each one of these elements and the corresponding critical values (Gurrera *et al.*, 2011).

Previously published diagnostic criteria have been based upon personal experience or literature reviews and a lack of agreement in the diagnosis of NMS has emerged when compared one with another (Gurrera, Chang and Romero, 1992). Moreover, previous studies have not provided methods for

evaluating the importance of each clinical feature relative to the others, making a distinguish only between major and minor elements or assigning equal weight to specific and non-specific findings (Levenson, 1985; Addonizio, Susman and Roth, 1986).

Conversely, these diagnostic criteria have been developed by an international and multidisciplinary group of clinicians and researchers with the aim to reflect a broad consensus among experts. Moreover, priority score and explicit critical values have been provided for each parameter, allowing for more reliable and specific diagnosis (Gurrera *et al.*, 2011). The latest edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) has included the international expert consensus (IEC) diagnostic criteria in the general description of NMS under the heading of clinical features (American Psychiatric Association, 2013).

More recently, IEC diagnostic criteria have been validated in the clinical population using DSM-IV-TR diagnostic schema as gold standard, since no biological marker is available for NMS (American Psychiatric Association, 2000; Gurrera *et al.*, 2017). Summing up IEC criteria priority points, a single cut-off score major or equal to 74 allowed for an accurate diagnosis (**Table 1**), resulting in a correct classification of more than 85% of cases with respect to the reference (Gurrera *et al.*, 2017). IEC criteria need to be further tested in more clinically heterogeneous populations, before they are routinely used in clinical practice (Gurrera *et al.*, 2011, 2017). However, adopting these generally accepted diagnostic criteria may be a step forward for both research and clinical management of patients (Velamoor, 2017).

Levenson, 1985	Pope *et al.*, 1986	Addonizio *et al.*, 1986	Caroff and Mann, 1993	DSM-IV, 1994	Adityanjee, Mathews and Aderibigbe, 1999	Gurrera *et al.*, 2011	
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. Leukocytosis	**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. Hyperthermia (≥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.	**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
						7. Hypermetabolism, defined as heart-rate increase (≥ 25% above baseline) AND respiratory-rate increase (≥ 50% above baseline)	5
						8. Negative work-up for infectious, toxic, metabolic, or neurologic causes	7

Table 1. Comparison of the main Diagnostic Criteria for Neuroleptic Malignant Syndrome. The table shows the main sets of Diagnostic Criteria for NMS published so far.

1.3.1 Differential diagnosis

Despite differences, all sets of criteria developed since the mid-1980s for NMS diagnosis underline that NMS is a diagnosis of exclusion and other medical conditions characterized by similar signs and symptoms should be ruled out before making a diagnosis, with special attention to those presenting with hyperthermia and/or muscle rigidity (Strawn, Keck and Caroff, 2007; Tse *et al.*, 2015). Indeed, numerous other medical conditions, being both drug-induced or not, can present with hyperthermia and associated muscle rigidity and all of these should always be considered and excluded before NMS is diagnosed (Oruch *et al.*, 2017; Ware, Feller and Hall, 2018; Jamshidi and Dawson, 2019; Simon, Hashmi and Callahan, 2020).

1.3.1.1 Non drug-induced disorders

Several medical conditions resembling NMS that are not due to drug exposure, and all of them should always be ruled out before making NMS diagnosis (Ware, Feller and Hall, 2018; Jamshidi and Dawson, 2019). These disorders include infections, brain structural pathologies, autoimmune disorders, endocrinopathies, and heatstroke (Caroff, 1980; Caroff and Mann, 1993). Patients with schizophrenia or mood disorders can develop malignant catatonia, a condition that can be indistinguishable from NMS (Sienaert, van Harten and Rhebergen, 2019).

In all of this cases, a revision of patient's history, a careful evaluation of any drugs taken and of the clinical picture are fundamental for differential diagnosis (Oruch *et al.*, 2017; Jamshidi and Dawson, 2019).

1.3.1.1.1 Infections

CNS infections, especially viral encephalitis, can be difficult to distinguish from NMS (Strawn, Keck and Caroff, 2007). Previous known viral illness, headaches, meningeal irritation, seizures, CSF abnormalities and neuroimaging studies can help in diagnosis and avoid delay in treatment of the infection (Strawn, Keck and Caroff, 2007; Simon, Hashmi and Callahan, 2020).

The risk of NMS may be increased in patients infected by HIV and other viruses that affect brain structures treated with neuroleptics. Differential among drug-induced effects and viral pathology may be difficult, however discontinuation of neuroleptic therapy is suggested (Caroff and Mann, 1993).

1.3.1.1.2 Brain structural pathologies

Brain lesions resulting from tumours, abscesses, stroke, or trauma can produce symptoms mimic NMS, probably due to dopaminergic pathways damage. History, neurologic examination, and brain imaging studies can help in the differential diagnosis, as specific pattern of brain injury has never been found in NMS (Caroff and Mann, 1993).

1.3.1.1.3 Autoimmune disorders

Systemic lupus erythematosus and miscellaneous connective tissue pathologies can present with elevation in body temperature, movement disorders, and mental status alterations, that may be confused with NMS, if the patient is on neuroleptic therapy (Caroff and Mann, 1993).

1.3.1.1.4 Endocrinopathies

Thyrotoxicosis and phaeochromocytoma should be considered in the differential diagnosis of NMS, as they can present with hyperthermia, tremor, tachycardia, hypertension, and sweating. However, they are rarely associated with muscle rigidity (Caroff and Mann, 1993).

1.3.1.1.5 Heatstroke

Heatstroke is a potentially fatal medical condition characterized by body temperature elevation and altered mental status, resulting from exposure to hot and humid environments and/or intense physical activity (Liu *et al.*, 2020).

Unlike NMS, heatstroke is not associated to muscle rigidity (Caroff and Mann, 1993; Tse *et al.*, 2015). However, both disorders share some clinical features and may be confused one with another in patients on antipsychotic treatment. By altering central heat-loss mechanisms, administration of antipsychotics increase the risk of heatstroke, predisposing patients to develop the so-called antipsychotic drug-related heatstroke (ADRHS) (Mann

et al., 2003). Alteration of central thermoregulation appears to be involved in the onset of both ADRHS and NMS. However, ADRHS appears as a drug-facilitated heatstroke in which antipsychotics increase vulnerability to environmental heat, whereas NMS represent a form of drug-induced heatstroke as excess heat seems to derive primarily from hypermetabolism of skeletal muscle caused by antipsychotic drug–induced extrapyramidal rigidity, independently of environmental temperature (Mann *et al.*, 2003).

1.3.1.1.6 Catatonia

Recognized since 1874, before the advent of psychopharmacological treatments, catatonia is a condition of marked psychomotor disturbance that can occur in the context of several mental disorders (e.g., schizophrenia, bipolar or depressive disorder) or medical conditions (e.g., tumours, head trauma, encephalitis) (Fink and Taylor, 2009; American Psychiatric Association, 2013). Catatonia presentation can be very variable, ranging from decreased motor activity or reduced psychosocial engagement to marked agitation and peculiar motor activity, with a potentially lethal progression of symptoms from mild or simple to malignant (Mann *et al.*, 1986; American Psychiatric Association, 2013). Malignant catatonia and NMS shares common symptoms such as fever, rigidity, autonomic instability, laboratory abnormalities, and response to treatment with both benzodiazepines and ECT (Mann *et al.*, 1986; Rosebush and Mazurek, 2010; Pileggi and Cook, 2016). Although semiology can be similar, usually there is no history of neuroleptic administration in the case of catatonia (Tse *et al.*, 2015). Otherwise, differentiation from NMS in neuroleptic-treated patients is more difficult and the two conditions may be indistinguishable in more than 20% of cases (Mann *et al.*, 1986; American Psychiatric Association, 2013). Thus, some investigators consider neuroleptic malignant syndrome to be a drug-induced form of malignant catatonia, but the topic remains considerably controversial (White, 1992; Fink, 1996; Northoff, 1996; Margetić and Margetić, 2010; Komatsu *et al.*, 2016).

1.3.1.2 Drug-induced disorders

Numerous drug-induced conditions may present symptoms similar to NMS (Strawn, Keck and Caroff, 2007). Differential diagnosis can be difficult,

however the drug history together with the time course of symptoms development can help in diagnosis (Jamshidi and Dawson, 2019). Differentiating among NMS and other drug-induced conditions presenting similar symptoms is important not only because the treatment may be different, but also because the diagnosis will affect how to approach restarting treatment with neuroleptics if necessary (Strawn, Keck and Caroff, 2007).

MH is considered in the differential diagnosis of NMS only in patients undergoing general anaesthesia, when both MH triggering agents and antipsychotics may be administered (Mann *et al.*, 2003). Although some symptoms appear to be similar, NMS does not occur intraoperatively, unlike MH, because anaesthetics and muscle relaxants used during surgery probably inhibit the central mechanisms leading to its onset (Mann *et al.*, 2003). Both syndromes are successfully treated with dantrolene, however only NMS responds to dopamine agonists and electroconvulsive therapy (ECT) (Mann *et al.*, 2003; Krause *et al.*, 2004).

Serotonin Syndrome (SS) is the most common condition that can be misdiagnosed as NMS, especially in its most severe presentations (Strawn, Keck and Caroff, 2007; Tse *et al.*, 2015; Pileggi and Cook, 2016; Simon and Keenaghan, 2020). A careful evaluation of the clinical picture can help in distinguishing the two syndromes. SS onsets more rapidly than NMS, usually appearing within 1 to 24 hours after introducing or changing a therapy that affects serotonin levels (Talton, 2020). SS patients are generally agitated and present myoclonus, that is a common and unique characteristic of SS, while NMS patients are usually mute and immobile (Paden MS, Franjic L, 2013). Moreover, SS can present with gastrointestinal disturbances such as diarrhoea, nausea, and vomiting, which are rarely seen in NMS (Pileggi and Cook, 2016). However, recent cases of SS presenting with symptoms commonly associated with NMS have been reported in literature (Mazhar *et al.*, 2016; Werneke *et al.*, 2016). This seems to suggest that sometimes conditions sharing both SS and NMS characteristics can present, especially when the offending agent or the combination of offending agents has both anti-dopaminergic and serotonergic activity (Scotton *et al.*, 2019). Cocaine and MDMA are substance of abuse usually associated with SS, since they

increase nor/epinephrine, dopamine and serotonin neurotransmission by inducing their release from the presynaptic axon terminal or inhibiting their re-uptake (Rusyniak and Sprague, 2005; Paden MS, Franjic L, 2013; Francescangeli *et al.*, 2019; Scotton *et al.*, 2019). However, cases presenting with clinical features resembling NMS have been reported, suggesting an overlap between the two syndromes (Daras *et al.*, 1995; Demirkiran, Jankovic and Dean, 1996; Wetli, Mash and Karch, 1996; Russell *et al.*, 2012). Common pathogenetic mechanisms have been proposed, since chronic cocaine use may affect dopaminergic neurotransmission either by dopamine depletion due to reuptake inhibition resulting in a reduction of released dopamine to the synaptic cleft or by a decrease in the number of postsynaptic dopamine receptors, to compensate for the overstimulation of the dopaminergic system coming from cocaine (Dackis and Gold, 1985; Volkow *et al.*, 1990; Daras *et al.*, 1995). Moreover, it has been observed that the increased stimulation of serotonin receptors inhibits dopamine release (Demirkiran, Jankovic and Dean, 1996; Steele, Keltner and McGuiness, 2011). When NMS and SS appear indistinguishable, benzodiazepines administration is suggested as the safest therapeutic choice (Rusyniak and Sprague, 2005).

Anticholinergic toxicity, a syndrome associated with the intake of anticholinergic medications, may resemble NMS presenting with confusion and fever. However, typical signs of this disorder include dry skin and normal muscular tone, contrasting with the diaphoresis and rigidity that are typical of NMS (Tse *et al.*, 2015; Jamshidi and Dawson, 2019).

Lastly, sudden discontinuation of CNS depressants such as alcohol, benzodiazepine or barbiturate may trigger tremors, confusion, hallucinations, tachycardia, and hypertension; however, hyperthermia is usually absent (Francescangeli *et al.*, 2019).

1.3.2 Management and treatment

The most important steps in NMS treatment are early recognition and prompt discontinuation of the potential offending agents (Mann *et al.*, 2003; Berman, 2011; Pileggi and Cook, 2016). The morbidity and mortality risks associated with NMS exceed the risk of untreated psychosis resulting from

abrupt withdrawal of neuroleptic therapy, indeed it is considered prudent to stop neuroleptic drugs even if the diagnosis of NMS is not conclusive (Berman, 2011; Tse *et al.*, 2015; Pileggi and Cook, 2016). Lithium, antidepressants and drugs with potent anticholinergic properties should be suspended, too (Mann *et al.*, 2003). However, the opposite is true in the case of dopamine agonist medication: sudden withdrawal of prodopaminergic medications can cause NMS or worsen its symptoms, while their continued use may have a protective role (Mann *et al.*, 2003; Berman, 2011; Pileggi and Cook, 2016).

1.3.2.1 Supportive treatment

After discontinuation of neuroleptics administration, the next key step in NMS treatment is the initiation of supportive management of vital functions and prevention of complications: cooling blankets and ice packs may be used to reduce hyperthermia, aggressive hydration is often necessary to prevent dehydration due to poor oral intake and sweating, acid-base alterations should be corrected maintaining a slightly alkalotic pH to promote the excretion of by-products of muscle breakdown and preventing renal failure, intubation and ventilatory support may be required to face respiratory failure (Mann *et al.*, 2003; Berman, 2011; Pileggi and Cook, 2016). Apart from the discontinuation of the offending drugs and the adoption of non-pharmacological measures, non-specific pharmacological treatment can be implemented (Tse *et al.*, 2015). The administration of calcium channel blockers antihypertensives to control labile blood pressure has been suggested because their effect may also be beneficial at the musculoskeletal level (Tse *et al.*, 2015; Pileggi and Cook, 2016). To prevent deep-vein thrombosis, pulmonary emboli and DIC resulting from muscle rigidity and immobilization, heparin may be indicated as prophylaxis (Mann *et al.*, 2003; Tse *et al.*, 2015).

1.3.2.2 Pharmacological treatment

The implementation of a specific pharmacological therapy for NMS remains controversial (Mann *et al.*, 2003; Tse *et al.*, 2015).

According to Shalev and co-workers, the protective role of specific drugs in the treatment of NMS is very low: summing up case reports published

between 1984 and 1989, they observed that the difference between mortality among patients treated with either one or more of these drugs (9.3%) and patients treated with only supportive therapy (13,5%) was not significant. Caution is needed in interpreting this observation, since it could result more from the overreporting of serious cases, rather than clinical reality (Shalev, Hermesh and Munitz, 1989). However, some authors agree and put caution in initiating drug therapy rapidly, suggesting to wait one to three days of observation ad support treatment before making the decision to use or not specific therapies, as patients may improve from supportive treatment alone (Pelonero, Levenson and Pandurangi, 1998). Indeed, Rosebush and colleagues reported that, in a cohort of 20 NMS patients, those who received specific pharmacological therapy had a longer course of illness and a greater incidence of morbid sequelae than those treated with supportive care alone, indicating a not useful role for specific pharmacological agents in the treatment of NMS (Rosebush, Stewart and Mazurek, 1991).

On the other hand, Rosenberg and Green observed that the addition of a specific drug treatment to conservative measures significantly reduces the time from the initiation of treatment to clinical improvement (Rosenberg and Green, 1989).

Actually, randomized controlled trials comparing specific pharmacological treatments with supportive care are lacking, so the arguments supporting the use of specific pharmacological treatments are based on theoretical reasons, case reports an experts' opinion (Mann *et al.*, 2003; Tse *et al.*, 2015; Pileggi and Cook, 2016; van Rensburg and Decloedt, 2019). Early recognition, prompt discontinuation of neuroleptics, and initiation of supportive medical treatment remain the mainstay of NMS management. These can be accompanied by specific pharmacological treatments, including benzodiazepines, dopaminergic agonists and dantrolene (Pelonero, Levenson and Pandurangi, 1998; Mann *et al.*, 2003). However, no specific drug appears to be universally effective, in fact the treatment of NMS should be individualized and based on the clinical presentation, even if some general guidelines have been developed (Strawn, Keck and Caroff, 2007;

Berman, 2011; Tse *et al.*, 2015; Pileggi and Cook, 2016; van Rensburg and Decloedt, 2019).

1.3.2.2.1 Benzodiazepines

Clinical reports suggest that benzodiazepines (BDZs) have been effective in improving symptoms and accelerate recovery from NMS, particularly in milder cases (Francis *et al.*, 2000; Yacoub and Francis, 2006). Their efficacy is explained by the reversal of the low functioning of the GABAergic system that contributes to NMS symptoms (Pileggi, Cook 2016).

However, cases of NMS in which BDZs had no or only transient clinical effect have been reported: Addonizio *et al*. reviewed 21 cases of benzodiazepines-treated NMS of which only 3 claimed efficacy, 8 experienced a temporary improvement of symptoms and 10 described no or unclear response (Addonizio, Susman and Roth, 1987). Moreover, Nielsen *et al*. observed that patients treated with BDZs were at more risk of developing NMS, but conclusions of a possible link between BDZs and the risk of developing NMS have to be conservative due to methodological limitations of the study design (Nielsen *et al.*, 2012).

Nonetheless, benzodiazepines are generally considered a reasonable first-line intervention in patients with NMS, above all in milder cases (Mann *et al.*, 2003; Strawn, Keck and Caroff, 2007).

1.3.2.2.2 Dopamine agonists

A reduced functioning of the central dopaminergic activity seems to play a major role in the onset of NMS, such that dopaminergic agents have been successfully used in treating the syndrome (Mann *et al.*, 2000; Strawn, Keck and Caroff, 2007; Pileggi and Cook, 2016).

Bromocriptine is an ergot derivative with potent agonist activity at the postsynaptic dopamine D_2 receptor and, since 1983, it has been used in NMS to reverse the blocking activity on this receptor attributed to neuroleptics (Pileggi and Cook, 2016). In 1989, Rosenberg and Green reviewed 67 NMS cases of which sufficient data to allow evaluations about response to specific pharmacological therapy were known and observed that the addition of

bromocriptine to supportive treatments significantly shortened the time from the initiation of treatment until the beginning of clinical improvement (1.03 ± 0.55 days) when compared with supportive treatments alone (6.80 ± 2.68 days) (Rosenberg and Green, 1989). Accordingly, analysing 98 case reports of NMS patients who were treated with bromocriptine, Sakkas and co-workers found that it significantly reduced mortality rate, either used alone or in combination with other drugs, compared with patients treated with supportive care only (Sakkas *et al.*, 1991). In contrast with these findings, Rosebush and co-workers reported that patients treated with bromocriptine alone ($n = 2$) or in combination with dantrolene ($n = 4$) had a longer course of disease and suffered more morbid sequelae that patients treated supportive measures only ($n = 12$) (Rosebush, Stewart and Mazurek, 1991). However, bromocriptine is generally considered effective and so its use is suggested in moderate to severe cases of NMS, with addition of dantrolene in the most serious cases (Strawn, Keck and Caroff, 2007; Pileggi and Cook, 2016; van Rensburg and Decloedt, 2019).

A further dopaminergic treatment described in literature for moderate to severe NMS is amantadine, whose action results mainly from both an increase in dopamine release and a reduction in its reuptake (Pileggi and Cook, 2016). In a review of the literature, Sakkas et *al*., found that amantadine was used in 32 cases of NMS in combination with other drugs and in other 17 cases alone, with a significative reduction in death rate among cases in which it was used in combination but only a trend in the same direction when used alone (Sakkas *et al.*, 1991). More recently, amantadine alone has been successfully used to treat NMS induced by haloperidol in a patients who had suffered a traumatic brain injury (Wilkinson, Meythaler and Guin-Renfroe, 1999). However, amantadine is generally suggested as an alternative to bromocriptine to treat moderate to severe NMS in combination to benzodiazepines or dantrolene, respectively (Pileggi and Cook, 2016; van Rensburg and Decloedt, 2019).

1.3.2.2.3 Dantrolene

Hyperthermia in NMS is probably due to neuroleptic-induced hypothalamic dopamine D_2 receptor antagonism with consequent impairment

in heat loss pathways combined with excessive heat production secondary to extrapyramidal muscle rigidity (Gurrera and Chang, 1996; Gurrera, 1999).

Dantrolene is the drug of choice for the specific treatment of malignant hyperthermia (MH), a rare but potentially fatal condition that presents as a hypermetabolic response to halogenated anaesthetic gasses and the muscle relaxant succinylcholine in genetically susceptible subjects (Watt and McAllister, 2020). Dantrolene is successfully used in MH patients to reduce core body temperature and peripheral muscle rigidity by inhibiting calcium release from the muscle sarcoplasmic reticulum (Krause *et al.*, 2004). Side effects may occur after administration of dantrolene: muscle weakness, local inflammatory phlebitis at the infusion site caused by the highly alkaline solution, impairment of the respiratory function and gastrointestinal discomfort are the side effects most frequently observed in MH patients treated with dantrolene (Brandom *et al.*, 2011). During dantrolene administration, hepatic function should be monitored, as cases of hepatic injuries have also been reported in association with its use (Chan, 1990).

Having shown his efficacy in treating body temperature elevation and muscle rigidity resulting from MH, dantrolene has been tried as treatment for NMS starting from 1981 (Delacour *et al.*, 1981; Shalev, Hermesh and Munitz, 1989). Dantrolene may be beneficial in reducing hyperthermia and contraction of skeletal muscles during NMS cases complicated by extreme temperature elevations and severe rigidity (Mann *et al.*, 2003; Pileggi and Cook, 2016; van Rensburg and Decloedt, 2019). It can be administered alone or in combination with benzodiazepines or dopamine agonists, but association with calcium-channel blockers should be avoided, as cardiovascular collapse can occur. However, controlled clinical trials are lacking and evidences supporting its use are based only on case reports (Mann *et al.*, 2003; Strawn, Keck and Caroff, 2007).

Addonizio and colleagues reviewed all cases of NMS in the English-language literature until 1987, identifying a total of 115 cases. Dantrolene was tried as a treatment for NMS in 20 cases, of which 15 responded to it and 5 did not (Addonizio, Susman and Roth, 1987). In 1989, Rosenberg and Green reviewed 67 cases of NMS observing that the addition of dantrolene to

supportive treatments significantly shortened the time from the initiation of treatment until the beginning of clinical improvement (1.72 ± 1.15 days) when compared with supportive treatments alone (6.80 ± 2.68 days) (Rosenberg and Green, 1989). A couple of years later, Sakkas and co-workers performed a case-controlled study to assess the effectiveness of dantrolene for NMS by analysing all known published cases and using as controls those cases not treated with specific drugs: the control group showed a mortality rate of 21%, while the dantrolene-treated group mortality was 8.6% (Sakkas *et al.*, 1991). More recently, Tsutsumi *et al*. reported that among 21 cases of NMS treated with dantrolene, the prognosis was generally good and no deaths occurred (Tsutsumi *et al.*, 1998).

On the other hand, some authors caution about dantrolene treatment of NMS. Rosebush and co-workers reported that patients treated with dantrolene alone ($n = 2$) or in combination with bromocriptine ($n = 4$) had a longer course of disease and suffered more morbid sequelae than patients treated supportive measures only ($n = 12$) (Rosebush, Stewart and Mazurek, 1991). Consistently, a review by Reulbach *et al*. of 271 cases of NMS reported in literature from 1980 to 2006 concluded that dantrolene used in combination with other drugs led to a prolongation of the time of remission. Furthermore, treatment of NMS with dantrolene as monotherapy seemed to be associated with a higher mortality rate (Reulbach *et al.*, 2007). However, it should be noted that pharmacological therapy tend to be reserved for severely ill patients, so these observations could result from an overrepresentation of serious cases, a delayed administration of the drug or an inadequate dosage (Shalev, Hermesh and Munitz, 1989; Strawn, Keck and Caroff, 2007).

1.3.2.2.4 Electroconvulsive Therapy

Performed under general anaesthesia, electroconvulsive therapy (ECT) is a therapeutic procedure that consists in delivering a small electric current to the brain (Ruth-Sahd, Rodrigues and Shreve, 2020). Though its mechanism of action is unclear, it has been shown to be effective in a variety of refractory disorders, including NMS (Davis, Caroff and Mann, 2000; Pileggi and Cook, 2016). ECT is usually considered as an therapeutic option, when other treatments have failed (Pileggi and Cook, 2016).

1.3.3 Outcome

A reduction in NMS mortality rate has been reported since its first description in 1960 to date: according to a review by Kellam that comprised more than 200 published cases of NMS, the syndrome resulted in a 76% mortality before 1970, a 22.7% mortality from 1970 to 1980 and a 14.9% mortality for the cases reported from 1980 onwards (Kellam, 1987). Among 1980 and 1987, about 10% out of 256 cases of NMS described in literature had a fatal outcome (Caroff and Mann, 1988). These observations were confirmed by Shalev and colleagues who reported a decrease in NMS mortality from about 25% before 1984 to 11.6% after 1984, probably thanks to increased awareness of the syndrome by physicians with consequent earlier recognition and treatment (Shalev, Hermesh and Munitz, 1989).

In fatal cases, death is more attributable to complications that can occur during the course of the disease, rather than to the pharmacological mechanisms that trigger NMS (Shalev, Hermesh and Munitz, 1989). Complications develop mainly as physiologic consequences of severe rigidity and immobilization: poor oral intake leads to dehydration, increasing the risk of rhabdomyolysis due to extreme muscle contraction, which in turn may lead to myoglobinuria and acute renal failure (Shalev, Hermesh and Munitz, 1989; Pelonero, Levenson and Pandurangi, 1998). Deep venous thrombosis and pulmonary embolism may occur as results of rigidity, immobilization and dehydration, too (Pelonero, Levenson and Pandurangi, 1998). Hyper-salivation combined with difficulty swallowing and mental status alterations can cause aspiration pneumonia and patients may need to receive ventilatory support (Pelonero, Levenson and Pandurangi, 1998; Oruch *et al.*, 2017). Among 53 NMS cases reviewed by Levenson, 10 patients (18.9%) needed ventilatory support (Levenson, 1985). Other causes of respiratory distress can include shock, neuroleptic-induced decreased chest wall compliance and necrosis of respiratory muscles due to rhabdomyolysis (Mann *et al.*, 2003). Myocardial infarction, DIC and sepsis have been reported as other possible complications of NMS (Levenson, 1985). Takotsubo cardiomyopathy, a reversible left ventricular syndrome, has been described as a result of autonomic nervous system dysfunctions (Oomura *et al.*, 2004). Autonomic

impairment can also lead to hypertensive crisis, hyperpyrexia, metabolic acidosis and coma (Oruch *et al.*, 2017).

Patients who survive NMS can experience morbid sequelae that persist for weeks to months after the resolution of the syndrome in a 3-10% of cases (Adityanjee, Sajatovic and Munshi, 2005). Post NMS neuromuscular abnormalities, including residual muscle rigidity, tremors and dystonia, have been commonly reported (Mann *et al.*, 2003; Adityanjee, Sajatovic and Munshi, 2005). Peripheral neuropathies with consequent sensory deficit and weakness may also occur after NMS, probably due to prolonged immobility (Anderson and Weinschenk, 1987; Roffe *et al.*, 1991; Adityanjee, Sajatovic and Munshi, 2005). Long-term cognitive sequalae are quite rare, but may be encountered in patients who survive NMS complicated by a marked hyperthermia or hypoxia (Levenson, 1985; Mann *et al.*, 2003; Adityanjee, Sajatovic and Munshi, 2005).

However, increased awareness of the syndrome by clinicians with consequent earlier recognition and treatment usually allow for NMS to fully resolve over the course of 3-14 days (Tse *et al.*, 2015).

1.3.4 Post-NMS management

Psychosis is a severe illness and restarting treatment with neuroleptics after recovery from NMS may be necessary (Pelonero, Levenson and Pandurangi, 1998).

Rechallenge is associated with a risk of developing NMS again as high as 30% (Caroff and Mann, 1988; Tsutsumi *et al.*, 1998). In the majority of cases, a different agent is chosen and the administration of a second-generation neuroleptic with a low D_2 receptor affinity is preferred for rechallenge (Pileggi and Cook, 2016; Oruch *et al.*, 2017). However, cases of successful rechallenge with the same neuroleptic that had caused the syndrome have been reported (Lally *et al.*, 2019). Moreover, according to a review of 41 reported cases of neuroleptics rechallenge after NMS, recurrence was not associated with the neuroleptic agent used (Wells, Sommi and Crismon, 1988). Recurrence of NMS may depend more on time elapsed between recovery and rechallenge. Evidence coming from case series analysis suggest

waiting a period of 5-14 days after resolution of a previous episode before reintroduction of neuroleptic therapy (Wells, Sommi and Crismon, 1988; Rosebush, Stewart and Gelenberg, 1989).

Nevertheless, most patients who require restarting neuroleptic treatment can be successfully treated, provided precautions are taken. For example, reports of previous episodes should be checked for accuracy; alternative medications should be considered; risk factors should be reduced; neuroleptic administration should be started at a low dose and titrated gradually with a carefully monitoring of the and patients for early signs of NMS (Strawn, Keck and Caroff, 2007).

1.4 Serotonin Syndrome

Serotonin syndrome (SS) is a potentially fatal syndrome that results from an augmented serotoninergic activity in both the peripheral (PNS) and central nervous system (CNS) (Scotton *et al.*, 2019). It is considered a spectrum disorder with manifestations ranging from mild gastrointestinal symptoms to more severe forms characterized by hyperthermia and muscle rigidity (Paden MS, Franjic L, 2013). SS typically occurs following exposure to antidepressant agents, such as serotonin agonists and/or tricyclic antidepressants (TCA), serotonin reuptake (SRI) or monoamine oxidase (MAOI) inhibitors, for which the incidence of use in adults in the United States has increased from 6.5% in 1999-2000 to 10.4% in 2009-2010 (Paden MS, Franjic L, 2013; Mojtabai and Olfson, 2014; Scotton *et al.*, 2019). However, also other classes of drugs may be related to SS, including herbal preparations based on ginseng and St. John's worth; analgesics, such as tramadol and other opioids; triptans, used in the treatment of migraine; the anticonvulsant valproic acid, and certain antiemetics, such as metoclopramide, since all of them affect serotonin concentration (Wooltorton, 2006; Takeshita and Litzinger, 2009; Paden MS, Franjic L, 2013). Moreover, also certain illicit drugs including cocaine, amphetamines, and MDMA, whose availability and use is increasing over years, can evoke the syndrome (Hall and Henry, 2006; Paden MS, Franjic L, 2013; Korpi *et al.*, 2015; Scotton *et al.*, 2019). Thus, SS is an issue of growing public health concern that clinicians and researchers should be aware of (Scotton *et al.*, 2019).

1.4.1 Historical background

Epidemics of "convulsive ergotism" characterized by muscle contractions, alterations in mental status, and fever were well reported in Europe from 1085 to 1927 due to the consumption of grain contaminated with ergot, an alkaloid produced by the *fungus Claviceps purpurea* that is now known as a causative agent of SS (Eadie, 2003). Thus, SS may have been a public health problem long before the more recent advent of psychopharmacology or the increasing availability and use of illicit drugs such as cocaine and amphetamines (Eadie, 2003; Scotton *et al.*, 2019).

However, it will take until 1960 for the first case report to be published describing a clinical picture attributable to SS, following the co-administration of L-tryptophan, serotonin's precursor, and an MAOI (Oates and Sjoerdsma, 1960). The term SS was first used in 1980 describing the motor and behavioural effects of serotonin in rats (Gerson and Baldessarini, 1980). Two years later, the first of many case reports and reviews describing SS in humans was published (Insel *et al.*, 1982; Scotton *et al.*, 2019).

1.4.2 Incidence

Generally, the incidence of SS is thought to parallel the growing number of serotonergic agents prescribed in clinical practice (Boyer and Shannon, 2005; Mojtabai and Olfson, 2014). Recently published data coming from a retrospective cohort study performed by analysing records retrieved from two large US claims databases, showed a syndrome incidence ranging from 0.19% to 0.07% of patients treated with serotoninergic medications in the period 2008-2013 (Nguyen *et al.*, 2017). However rigorous epidemiological data regarding SS are not available, as it is a relatively rare disorder with a variable presentation that is often under-diagnosed and under-reported by clinicians, especially as regards mild cases (Scotton *et al.*, 2019).

1.4.3 The serotonin signalling pathway and the pathophysiology of SS

Serotonin (5-Hydroxytryptamine, 5-HT) is a neurotransmitter playing a major role in both the CNS and in the periphery (Brunton, Hilal-Dandan and Knollmann, 2017). In the CNS, 5-HT is synthesized by cells of the raphe nuclei positioned in the midline of brainstem. Axons from the serotonergic neurons in this region project into most of the brain cortical and subcortical structures (hypothalamus, thalamus, cerebral cortex, and cerebellum) regulating sleep, attention, affective behaviour, temperature, appetite, and sexual behaviour, whereas projections to spinal cord modulate motor tone and nociception (**Figure 8**) (Saper, 2000; Brunton, Hilal-Dandan and Knollmann, 2017). Peripheral 5-HT is synthesized by enterochromaffin cell located in the gastric mucosa and it is involved in gastrointestinal (GI) motility and in many cardiovascular functions, including vasoconstriction/vasodilation and

platelets aggregation (Brunton, Hilal-Dandan and Knollmann, 2017). Platelets do non synthetise 5-HT, but they express SERT and are able to uptake and store 5-HT from circulation. Initial platelets aggregation induces 5-HT release that stimulates continued platelet aggregation and constriction of the blood vessels during haemostasis by activation of platelet and vascular smooth muscle $5HT_{2A}$ receptors (Ni and Watts, 2006; Brunton, Hilal-Dandan and Knollmann, 2017). SERT inhibition by SSRIs may reduce platelet 5-HT increasing time for haemostasis (Ni and Watts, 2006). Thus, caution should be used in prescribing these medications to patients at risk for bleeding, such as those on anticoagulants therapy (Scotton *et al.*, 2019). Exaggerated increase of extra-cellular 5-HT may explain the occurrence of DIC as complication of severe SS (Miller *et al.*, 1991; Scotton *et al.*, 2019).

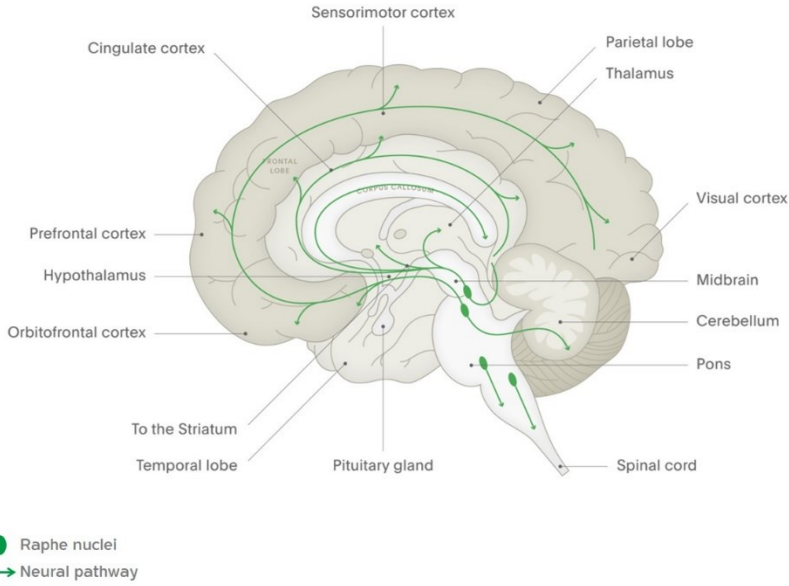

Figure 8. Brain serotonin pathways. Within CNS serotonin is synthesized by the cells of raphe nuclei in the brainstem. From this region, serotonergic projections innervate most of the brain cortical and subcortical structures regulating sleep, attention, affective behaviour, temperature, appetite, and sexual behaviour. Serotonergic projections to spinal cord modulate motor tone and nociception (Saper, 2000; Brunton, Hilal-Dandan and Knollmann, 2017). Adapted from Lundbeck Institute Campus (institute.progress.im).

5-HT is synthesized from the essential amino acid L-tryptophan that is actively transported into the brain by carrier protein LAT1, a transporter that also carries other neutral amino acids. Thus, brain concentration of tryptophan is related not only to its plasma concentration but also to the plasma concentrations of these other amino acids (Brunton, Hilal-Dandan and Knollmann, 2017). In rare instances, overabundance of L-tryptophan coming from the diet may cause SS, if combined with the administration of serotonin drugs (Fernstrom, 2012). The conversion of L-tryptophan in L-5-hydroxytryptophan by the enzyme tryptophan hydroxylase (TPH) is the initial and rate-limiting step in 5-HT synthesis. Subsequently, L-5-hydroxytryptophan is converted to 5-HT by aromatic L-amino acid decarboxylase enzyme (AADC). Once synthesized, 5-HT is deposited in presynaptic vesicles by the nonspecific amine carrier VMAT2, until the depolarization of the presynaptic terminal induces its release into the synaptic cleft. The release of 5-HT in the synaptic cleft induces several effects by binding to 5-HT postsynaptic receptors, whereas binding to presynaptic auto-receptors modulates further release of the neurotransmitter in the synaptic cleft. Effects of serotonin are terminated by re-uptake into the presynaptic axon terminal via re-uptake transporter (SERT) and metabolism by the enzyme monoamine oxidase (MAO). Of the two existing isoforms of MAO, MAO-A preferentially metabolizes 5-HT by converting it to an acetaldehyde intermediate that is further oxidized to 5-hydroxyindoleacetic acid (5-HIAA) by an aldehyde dehydrogenase. The acetaldehyde intermediate can also be reduced to form the alcohol 5-hydroxytryptophol, but this alternative pathway for serotonin metabolism is normally insignificant (Brunton, Hilal-Dandan and Knollmann, 2017).

So far, fourteen 5-HT receptor subtypes organized in seven subfamilies, from 5-HT_1 to 5-HT_7, have been described (Brunton, Hilal-Dandan and Knollmann, 2017).

The 5-HT_1 subfamily comprises five G-protein coupled receptors that inhibit adenylyl cyclase: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}. The $5HT_{1A}$ and $5HT_{1B/1D}$ receptors act as auto-receptors modulating the release of serotonin in the synaptic cleft. $5HT_{1A}$ receptor is located also postsynaptically

(**Figure 9**). The role of the 5-HT_{1E} and 5-HT_{1F} receptors has not been clarified, so far (Brunton, Hilal-Dandan and Knollmann, 2017).

The 5-HT_2 subfamily consists of three receptors that activates phospholipase C via G_q: 5-HT_{2A}, 5-HT_{2B}, 5HT_{2C}. High densities of 5HT_{2A} receptor are found in the CNS, in platelets and smooth muscle cells (Brunton, Hilal-Dandan and Knollmann, 2017; Francescangeli *et al.*, 2019).

The 5-HT_3 receptor is the only 5-HT receptor that functions as a ligand-gated ion channel and it is involved in emesis response, indeed 5-HT_3 antagonists such as ondansetron are approved as antiemetics (Brunton, Hilal-Dandan and Knollmann, 2017).

The 5-HT_4 receptor activates adenylate cyclase via G_s. The activation of 5-HT_4 receptor increases secretion and GI motility (Brunton, Hilal-Dandan and Knollmann, 2017; Scotton *et al.*, 2019).

The 5-HT_5 receptors are G-protein coupled receptors that inhibit adenylyl cyclase. Humans only express functional 5-HT_{5A}, whereas 5-HT_{5B} is not functional. Widely expressed in the brain, its function is related to circadian rhythms and cognition (Brunton, Hilal-Dandan and Knollmann, 2017).

The 5-HT_6 and 5-HT_7 are both G-protein coupled receptors that activate adenylyl cyclase. Both are widely expressed in the CNS. The 5-HT_6 is abundant in cortical, limbic, and extrapyramidal regions suggesting that it could be involved in motor control and cognition, whereas 5-HT_7 is supposed to be involved also in the relaxation of smooth muscle in blood vessels and GI tract (Brunton, Hilal-Dandan and Knollmann, 2017). Recent studies have focused on these receptors as targets for drugs to use in management of Alzheimer disease or antidepressants. However, no drug that selectively targets these receptors is available for therapeutic purposes, so far (Brunton, Hilal-Dandan and Knollmann, 2017; Scotton *et al.*, 2019).

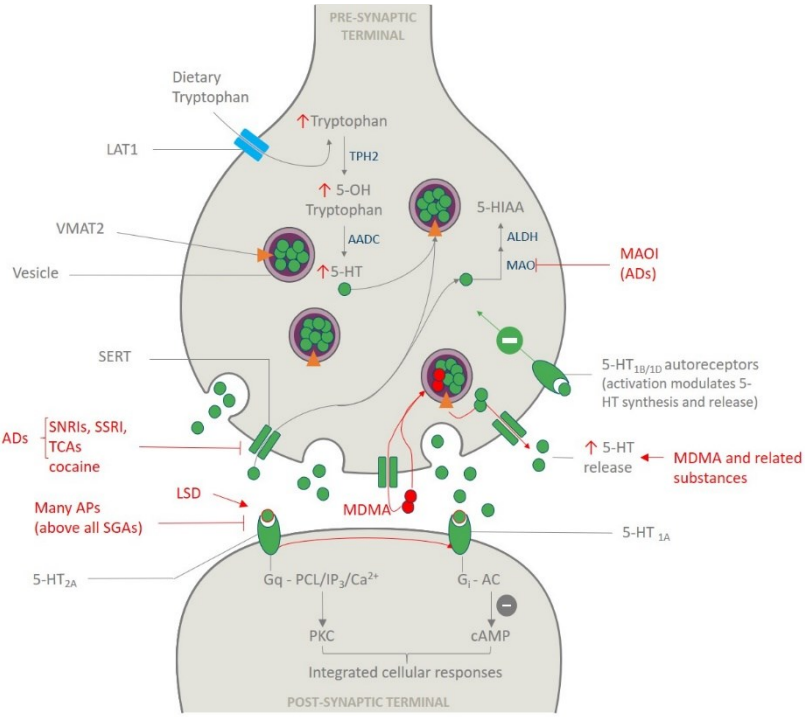

Figure 9. Schematic representation of a serotonergic synapse. SS is caused by hyperstimulation of post-synaptic 5-HT receptors. Mechanisms that may evoke the syndrome are shown in red: overabundance of L-tryptophan, as well as MAO inhibition, increases levels of presynaptic 5-HT; SERT inhibition by therapeutic drugs commonly used to treat depression or substances of abuse such as cocaine lead to increased synaptic levels of 5-HT; MDMA and related substances increase the release of 5-HT in the synaptic cleft; endogenous (5-HT) or exogenous (for example, LSD) agonists stimulates post-synaptic 5-HT receptors; antagonism of postsynaptic 5-HT_{2A} receptor by APs (above all SGAs) in the presence of SERT inhibition receptor is supposed to enhance stimulation of postsynaptic 5-HT_{1A} receptor triggering the onset of the syndrome.

Abbreviations: 5-HIAA, 5-hydroxy indole acetic acid; 5-HT, 5-hydroxytryptamine (serotonin); 5-HT_{1A}, 5-hydroxytryptamine receptor 1A; 5-HT_{2A}, 5-hydroxytryptamine receptor 2A; AADC, L-aromatic amino acid decarboxylase; AC, adenylyl cyclase; ADs, antidepressants; ALDH, aldehyde dehydrogenase; APs, antipsychotics; cAMP, cyclic adenosine monophosphate; LAT1, large neutral amino acid transporter 1; LSD, lysergic acid diethylamide; MAO, monoamine oxidase; MAOI, monoamine oxidase inhibitor; MDMA, 3,4-methylenedioxymethamphetamine; SGAs, second generation antipsychotics; SERT, serotonin re-uptake transporter; SNRIs, serotonin-norepinephrine re-uptake inhibitors; SSRIs, selective serotonin re-uptake inhibitors; TCAs, tricyclic antidepressants; TPH2, tryptophan hydroxylase 2; VMAT2, vesicular monoamine transporter 2.

Although no single receptor can be considered responsible for the onset of SS, agonism of 5-HT_{2A} receptor seems to play a major role in the onset of most severe forms of the syndrome (Francescangeli *et al.*, 2019). Additional subtypes of serotonin receptors, such as 5-HT_{1A} may contribute by saturation of all receptor subtypes due to augmented concentrations of serotonin receptors agonist in the synaptic cleft (Boyer and Shannon, 2005).

1.4.4 Causative agents

SS is a potentially fatal syndrome resulting from increased serotonin activity in both the central and the peripheral nervous system (Scotton *et al.*, 2019). Therefore, any drug capable of stimulating the activity of serotonin receptors can potentially evoke the syndrome (Boyer and Shannon, 2005). There are various pharmacological mechanisms through which this can happen: augmented serotonin synthesis, increased serotonin release, reduced serotonin metabolism, inhibition of serotonin reuptake, and activation of serotonergic receptors. However, in most cases multiple of these need to be triggered simultaneously for the syndrome to begin (Isbister and Buckley, 2005). Indeed, SS typically occurs after the addition of a further serotonergic agent to the therapeutic regimen (Paden MS, Franjic L, 2013).

1.4.4.1 Medications

Serotonin is synthesized from the essential amino acid L-tryptophan. Increased serotonin synthesis due to the overabundance of the precursor molecule from the diet and reduced metabolism of serotonin due to MAOI administration can both trigger SS (Fernstrom, 2012; Francescangeli *et al.*, 2019). Indeed, the first publication reporting a clinical picture attributable to SS described the coadministration of L-tryptophan with a MAOI inducing neurologic alterations (Oates and Sjoerdsma, 1960). Serotonin metabolism may also be affected by an altered functionality of the cytochrome P450, the major enzyme involved in drug metabolism, accounting for about 75% (Guengerich, 2004; Francescangeli *et al.*, 2019). Certain selective serotonin reuptake inhibitors (SSRIs) and antibiotics inhibit a number of CYP isoenzymes important for the metabolism of SSRI itself and other serotonergic drugs, resulting in an increased stimulation of serotonin

receptors that may trigger the onset of the syndrome (Mitchell, 1997; Lee, Franz and Goforth, 2009; Francescangeli *et al.*, 2019).

Certain drugs can cause SS by increasing serotonin concentrations at the synaptic cleft without altering its synthesis or metabolism. Antidepressant agents such as SSRIs, serotonin and noradrenaline reuptake inhibitors (SNRIs), and tricyclic antidepressants (TCAs) affect serotonin activity by inhibition of serotonin re-uptake transporter protein (SERT) (**Figure 10**) (Francescangeli *et al.*, 2019). Other classes of drugs that affect the activity of serotonin by inhibition of its re-uptake include the analgesic tramadol and other synthetic opioids (such as meperidine, methadone, dextromethorphan, and propoxyphene). They may evoke SS when taken in combination with other serotonergic medications or when administered alone in high doses (Mitchell, 1997; Paden MS, Franjic L, 2013; Francescangeli *et al.*, 2019).

Certain medications have serotonin receptor agonist activity. Triptans, a class of drugs used in management of migraine, act as 5-hydroxytriptamine receptor 1B (5-HT_{1B}) and 5-hydroxytriptamine receptor 1D (5-HT_{1D}) agonists (Wooltorton, 2006; Paden MS, Franjic L, 2013).

Contrary to the classical theory, that sees 5-HT_{2A} receptor agonism as a significant contributor to SS onset, a recent bioinformatic analysis has shown an association between SGA and SS. The authors suggested that the antagonistic activity exerted by SGAs on 5-HT_{2A} receptors in the presence of SERT inhibition causes a deviation of the elevated levels of serotonin on the 5-HT_{1A} receptor triggering the onset of the syndrome (Racz *et al.*, 2018; Scotton *et al.*, 2019). Consistently, a recent meta-analysis of 299 SS cases reported that 3% of them were associated with SGA as a decisive trigger. In this meta-analysis 14.0% of cases showed symptoms commonly associated with NMS. This seems to suggest that, when the offending agent or combination of offending agents has both anti-dopaminergic and serotonergic activity, the resulting syndrome might share both SS and NMS characteristics (Werneke *et al.*, 2016; Scotton *et al.*, 2019).

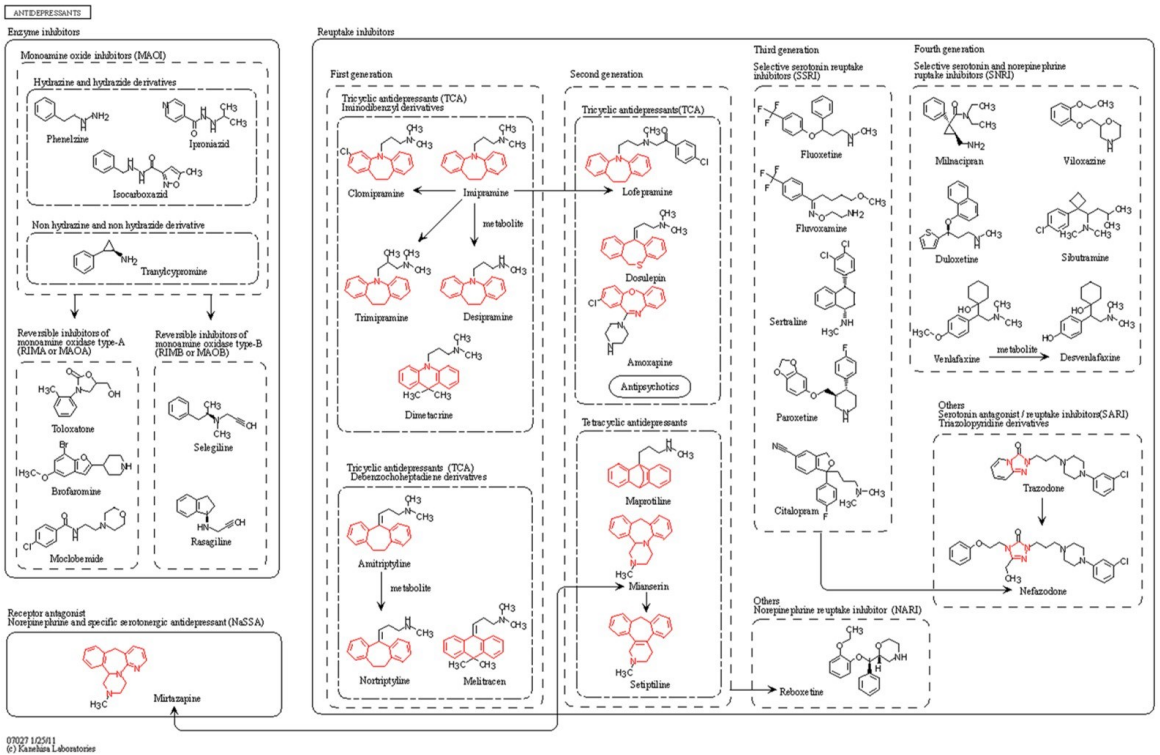

Figure 10. Antidepressants. Antidepressant drugs, including agents that may cause serotonin syndrome, are shown. Arrows indicate links among drug structures. Image obtained from: https://www.genome.jp/dbget-bin/www_bget?map07027.

Finally, the mood stabilizer lithium enhances antidepressant effectiveness by increasing the sensitivity of the postsynaptic serotonin receptors to their endogenous agonist without affecting the concentration of serotonin itself. So far, its mechanism of action remains unclear, however it has been frequently associated with the onset of SS (Mitchell, 1997; Francescangeli *et al.*, 2019).

1.4.4.2 Substances of abuse

Not only medications but also certain drugs of abuse have a potential risk of evoking SS (Scotton *et al.*, 2019).

The drug of abuse lysergic acid diethylamide (LSD) has agonist activity at 5-HT_1 and 5-HT_2 receptors (Nichols, 2016). Cocaine is a dopamine, norepinephrine, and serotonin reuptake inhibitor. Moreover, it also blocks voltage-dependent sodium channels, producing local anaesthesia (Korpi *et al.*, 2015; Drake and Scott, 2018).

In addition to LSD and cocaine, also novel psychoactive substances (NPSs) can produce SS (Scotton *et al.*, 2019). NPSs are new substances often synthetised as analogues of known drugs of abuse prohibited under current laws, such as amphetamines and cannabis (King and Kicman, 2011). NPSs are an increasing public health problem because of their growing availability and use by population: in 2014, 101 NPSs were identified by the European Union early warning system for the first time, and by the end of 2019 the NPSs under monitoring by European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) were 790, 53 of which were reported for the first time in Europe in 2019 (EMCDDA, 2015, 2020). Most NPSs affect levels of dopamine, serotonin, and noradrenaline in the CNS by inhibiting their re-uptake in the pre-synaptic terminal mediated by dopamine transporter DAT, serotonin re-uptake transporter SERT, and noradrenaline transporter NET (Scotton *et al.*, 2019). Among NPSs, ecstasy has been consistently associated with SS, depending on its serotonergic effects (Liechti, Kunz and Kupferschmidt, 2005). Ecstasy is the popular name for the compound 3,4-methylenedioxymethamphetamine (MDMA), a derivative of methamphetamine and its predecessor compound amphetamine, which constitutes one of the most common NPSs used in Europe (EMCDDA, 2020).

Indeed, MDMA binds to DAT, NET, and SERT but it shows higher affinity for SERT (Korpi *et al.*, 2015). It is taken up into the presynaptic neuron via SERT, resulting in increased synaptic levels of serotonin due to competitive reuptake inhibition. Moreover, it induces neurotransmitter efflux from synaptic vesicles, targeting the vesicular monoamine transporter VMAT2 that is responsible for packaging serotonin into vesicles once it is synthesized and facilitating serotonin release by SERT (**Figure 11**) (Korpi *et al.*, 2015; Francescangeli *et al.*, 2019).

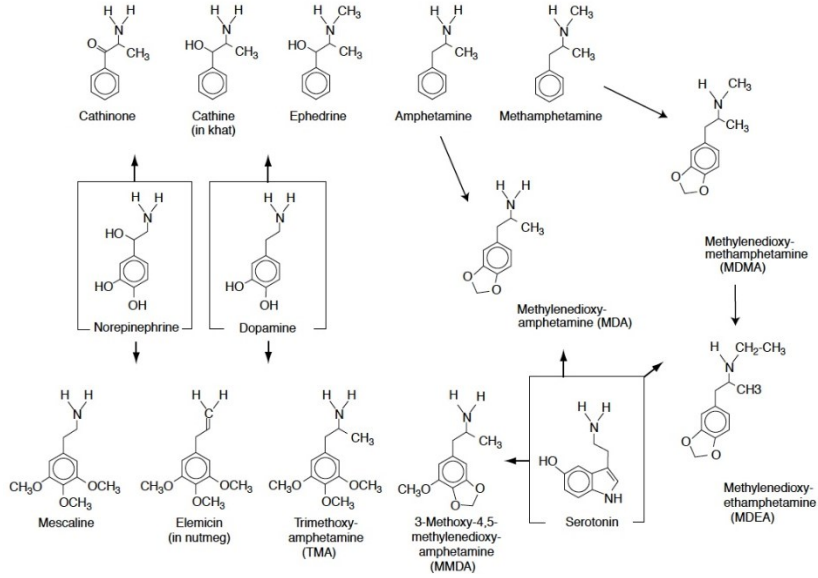

Figure 11. Chemical structures of MDMA and related compounds. Chemical structures of MDMA and related compounds are shown together with those of dopamine, norepinephrine, and serotonin. Similarities of structure are indicated by arrows. Image obtained from Kalant, 2001.

1.4.5 Risk factors

So far, no specific risk factors for the development of SS have been identified. However, it is possible to consider certain demographic, pharmacological, and genetic elements that could increase the likelihood of

developing the syndrome (Paden MS, Franjic L, 2013; Francescangeli et al., 2019; Scotton et al., 2019).

1.4.5.1 Demographic risk factors

SS has been identified in patients of all age groups, from new-borns to elderly, but older patients may be at greater risk of developing the syndrome, since many of the drugs able to evoke SS are commonly prescribed in the geriatric population (Mason, Morris and Balcezak, 2000; Isbister *et al.*, 2001; Laine *et al.*, 2003; Bobo *et al.*, 2019; Francescangeli *et al.*, 2019).

1.4.5.2 Pharmacological risk factors

Although SS has been described also following an increase in the therapeutic doses or after overdose of a single drug, the most common cause of SS is polypharmacy, with the combination of serotonergic drugs with MAOIs being especially dangerous, causing often severe SS (Gill, LoVecchio and Selden, 1999; Isbister *et al.*, 2004; Francescangeli *et al.*, 2019; Scotton *et al.*, 2019).

1.4.5.3 Genetic risk factors

Different patients may develop SS at different drug doses and/or combinations, suggesting underlying genetic factors may influence susceptibility (Scotton *et al.*, 2019). Genetic factors related to SS susceptibility could include variants in drug metabolizing enzymes such as cyto-chrome P450 2D6, and the gene *HTR2A* encoding for 5-HT_{2A} receptor (Murphy *et al.*, 2003; Scotton *et al.*, 2019).

A prospective, double-blind, randomized pharmacogenetic study compared treatment outcomes with the SSRI paroxetine and the non-SSRI antidepressant mirtazapine in 246 elderly patients with major depression (Murphy *et al.*, 2003). Results showed that the *HTR2A* 102 T/C variant (rs6313, according to Ensembl Release 101: G/A, forward strand) had a major effect on paroxetine-related side effects, with significantly more discontinuations due to severe adverse effects for paroxetine-treated patients carrying C/C genotype than those carrying the T/C and T/T genotypes. No relationship was found between *HTR2A* 102 T/C genotype and side effects

due to mirtazapine assumption. However, mirtazapine enhances serotonergic and noradrenergic transmission in the CNS by inhibiting presynaptic inhibitory receptors on noradrenergic and serotonergic neurons so that neurotransmitter release in the synaptic cleft is increased, but it only enhances serotonergic transmission via the 5-HT_{1A} receptor since it blocks 5-HT_2 and 5-HT_3 receptors, thus probably nullifying the effects of any functional 5-HT_{2A} variation (Murphy *et al.*, 2003; Francescangeli *et al.*, 2019). Another case report described a patient carrying C/C genotype who developed SS while taking the MAOI phenelzine alone (Lattanzi *et al.*, 2008). The *HTR2A* 102 T/C is a synonymous variant, thus it does not result in any aminoacidic substitution within the receptor protein. The effect found on paroxetine-induced adverse effects may be related to linkage disequilibrium with other variants modulating receptor functionality (Murphy *et al.*, 2003). However, contrary findings come from a study by Cooper and colleagues, who found no significant association between SS development and C/C genotype (Cooper *et al.*, 2014).

Variations in drug metabolism by CYPs have also been proposed to play a role in SS susceptibility (Kaneda *et al.*, 2002; Lorenzini *et al.*, 2012; Piatkov, 2017). A case report described SS occurring in a patient taking fluoxetine alone who was found to carry a loss-of-function *CYP2D6* genotype, as well as being heterozygous for an allele of *CYP2C19* resulting in poor metabolizer phenotype (Piatkov, 2017). Another case report describes the development of SS in a man after receiving a unique dose of paroxetine (Kaneda *et al.*, 2002). The *CYP2D6* allele analysis showed that this patient was homozygous for an allele that has been associated with relatively low in vivo cytochrome P450 2D6 activity (Kaneda *et al.*, 2002). However, the role played by polymorphisms of genes encoding CYPs in SS onset is further complicated by the numerous pharmacologic CYP inducers and inhibitors used in clinical practice, such as drugs for HIV treatment. Indeed, one case describing the development of SS in an HIV patient treated with antiretroviral medications which are known inhibitors of cytochrome P450 2C19 and cytochrome P450 3A4 and escitalopram has been reported. *CYP2D6* and *CYP2C19* genotyping showed that this patient carried alleles associated with poor enzymatic activity (Lorenzini *et al.*, 2012).

These findings suggest a putative role of *HTR2A* 102 T/C variant in the onset of SS, while large-scale studies are needed to investigate the relation between polymorphisms of genes encoding CYPs and SS onset (Francescangeli *et al.*, 2019; Ortiz *et al.*, 2020).

1.4.6 Clinical features

SS is a spectrum disorder that can present with milder symptoms of gastrointestinal disturbance to more severe forms characterized by hyperthermia and muscle rigidity (Paden MS, Franjic L, 2013). The onset of the syndrome is typically rapid occurring a few hours after serotonergic drugs intake (Simon and Keenaghan, 2020). Full-blown serotoninergic syndrome present with mental status alteration (agitation, confusion), enhanced autonomic (hyperthermia, tachycardia) and neuromuscular activity (manifesting as ankle and/or ocular clonus, hyperreflexia, and myoclonus) (Paden MS, Franjic L, 2013; Francescangeli *et al.*, 2019; Jamshidi and Dawson, 2019). Myoclonus is a characteristic manifestation of SS and helps in differential diagnosis from NMS (Simon and Keenaghan, 2020). If not recognized and treated promptly, symptoms may rapidly progress to muscle rigidity, rhabdomyolysis, hyperthermia major than 40°C, seizures, coma, and death (Francescangeli *et al.*, 2019).

1.4.7 Diagnostic criteria

Confirmatory laboratory tests for SS diagnosis are lacking, so diagnosis is based on evaluation of the clinical picture and compatible medical history, together with the exclusion of other disorders that may present with similar signs and symptoms (Paden MS, Franjic L, 2013; Francescangeli *et al.*, 2019; Simon and Keenaghan, 2020). So far, three main set of criteria have been proposed for SS diagnosis: the Sternbach, Radomski, and Hunter Criteria (**Table 2**) (Sternbach, 1991; Radomski *et al.*, 2000; Dunkley *et al.*, 2003). The first set of diagnostic criteria was developed in 1991 by Sternbach analyzing 38 SS cases in human patients to determine the most frequently reported clinical features of the syndrome (Sternbach, 1991). The main limitation of these diagnostic criteria regards the fact that they make possible to diagnose SS diagnosis based only on mental status alteration, a

characteristic common to many conditions (Francescangeli *et al.*, 2019). In 2000, Radomski and colleagues revisited Sternbach's diagnostic criteria based on the description of SS cases between 1995 and 2000 (Radomski *et al.*, 2000). So far, the Hunter Criteria are considered the gold standard for diagnosing the syndrome (Francescangeli *et al.*, 2019). Published by Dunkley *et al*. in 2003, this set of criteria was developed using a toxicology database called the Hunter Area Toxicology Service, which included patients who had taken at least one serotonergic drug in overdose and considering the symptoms recurring more frequently among them (Dunkley *et al.*, 2003; Francescangeli *et al.*, 2019). These criteria are considered the most accurate, however they may not be adequate for diagnosis of milder forms of SS (Simon and Keenaghan, 2020).

	Sternbach Criteria (Sternbach, 1991)	Radomski Criteria (Radomski *et al.*, 2000)	Hunter Criteria (Dunkley *et al.*, 2003)
Inclusion Criteria	Exposure to serotonergic agent	Exposure to serotonergic agent	Exposure to serotonergic agent
Exclusion Criteria	Presence of other aetiologies and/or addition of neuroleptic medication	Presence of other aetiologies and/or addition of neuroleptic medication	/
Signs and symptoms	At least three of the following: 1. Mental status alteration (confusion, hypomania); 2. Agitation; 3. Myoclonus; 4. Hyperreflexia; 5. Diaphoresis; 6. Shivering; 7. Tremor; 8. Diarrhoea; 9. Incoordination; 10.Fever.	Four major or three major plus two minor signs/symptoms. **Major:** 1. Altered consciousness; 2. Elevated mood; 3. Semicoma/coma; 4. Myoclonus; 5. Tremor; 6. Shivering; 7. Rigidity; 8. Hyperreflexia; 9. Fever; 10.Sweating. **Minor:** 1. Restlessness; 2. Insomnia; 3. Incoordination; 4. Dilated pupils; 5. Akathisia; 6. Tachy/Dyspnea 7. Diarrhoea; 8. Hypertension/Hypotension.	Any of the following combinations of signs and symptoms: 1. Spontaneous clonus alone or 2. Inducible clonus AND agitation or diaphoresis or 3. Ocular clonus AND agitation or diaphoresis or 4. Tremor AND hyperreflexia or 5. Hypertonicity AND body temperature > 38°C AND ocular clonus or inducible clonus.

Table 2. Main sets of criteria developed for SS diagnosis. The table shows Sternbach, Radomski, and Hunter Criteria for SS diagnois. Adapted from Francescangeli *et al.*, 2019.

1.4.8 Differential diagnosis

Milder presentations of SS are often overlooked or attributed to flu or food poisoning, if gastrointestinal symptoms are present (Simon and Keenaghan, 2020). Conversely, severe cases can be diagnosed as NMS (Simon and Keenaghan, 2020). However, usually SS develops more rapidly than NMS. Moreover, SS patients are generally agitated and present myoclonus, that is a common and unique characteristic of SS, while NMS patients are usually mute and immobile (Paden MS, Franjic L, 2013).

Anticholinergic toxicity, a syndrome associated with the intake of anticholinergic medications, may resemble SS presenting with agitation, confusion, and fever (Francescangeli *et al.*, 2019). However, typical signs of this disorder include dry skin and reduced gastrointestinal motility, contrasting with the diaphoresis and excessive gastrointestinal motility that are typical of SS (Francescangeli *et al.*, 2019; Jamshidi and Dawson, 2019). Moreover, in anticholinergic toxicity muscular tone and reflexes are normal (Scotton *et al.*, 2019). Malignant Hyperthermia present in genetically susceptible patients following exposure to triggering agents such as halogenated anaesthetic gasses and succinylcholine, resulting in severe hyperthermia and muscle rigidity. Absence of clonus and hyperreflexia and extreme muscle rigidity are distinguishing clinical feature from SS (Francescangeli *et al.*, 2019). Abrupt discontinuation of CNS depressants such as alcohol, benzodiazepine or barbiturate may trigger tremors, confusion, hallucinations, tachycardia, and hypertension, however hyperthermia is absent (Francescangeli *et al.*, 2019). Lastly, non-drug induced causes of hyperthermia and muscle rigidity, such as CNS lesions or infections or tetanus, should be excluded before making SS diagnosis (Jamshidi and Dawson, 2019; Scotton *et al.*, 2019).

1.4.9 Management and treatment

Discontinuation of the triggering agent as soon as SS is suspected, together with supportive therapy based on hydration, body cooling, and mechanical ventilation for severe cases plays a major role in the treatment of the syndrome (Paden MS, Franjic L, 2013). Myoclonus, hyperreflexia, and

seizures can be treated with benzodiazepines, that are non-specific serotonin antagonists (Paden MS, Franjic L, 2013). Cyproheptadine, a nonselective 5-HT_{1A} and 5-HT_{2A} antagonist, is considered the specific antidote for serotonin syndrome and its administration should be considered in moderate cases; however, its use has been criticized since the evidence for differential outcomes between patients receiving cyproheptadine and those receiving supportive care alone has not been considered substantial (Graudins *et al.*, 1998; Gillman, 1999; Paden MS, Franjic L, 2013; Francescangeli *et al.*, 2019). Some evidence support the administration of the atypical antipsychotics olanzapine and chlorpromazine to antagonize 5-HT_{2A} activity, but its use is discouraged due to concerns regarding the risk of hypotension and NMS (Boyer and Shannon, 2005). Patients with severe hyperthermia require immediate reduction of muscular activity by administration of nondepolarizing agents such coronium to induce paralysis, followed by mechanical ventilation (Boyer and Shannon, 2005). However, the onset of SS should ideally be prevented by implementing safer prescribing practices, such as using the lowest drug dose that effectively treats symptoms and avoiding polypharmacotherapy, and keeping the most at-risk patients under close medical supervision (Boyer and Shannon, 2005; Foong *et al.*, 2018).

1.4.10 Outcome

In milder cases prognosis is generally good. Indeed, if the syndrome is promptly recognized, the causative agent is discontinued and adequate treatments are implemented, serotonin syndrome typically resolve within 24 hours (Boyer and Shannon, 2005; Scotton et al., 2019) . However, symptoms may progress to muscle rigidity, hyperthermia major than 40°C, seizures, respiratory distress, multiorgan failure, DIC, cardiovascular collapse and death that has been reported in a 2% to 12% of cases (Miller et al., 1991; Paden MS, Franjic L, 2013; Francescangeli et al., 2019).

2 MATERIALS AND METHODS

2.1 Study design

A case-control study nested on a prospective cohort was conducted with the aim of identifying genetic variants associated to Neuroleptic Malignant Syndrome (NMS). The cohort study is on-going at the 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), where Drs Valeria Petrolini and Carlo Locatelli identify new NMS and NMS-like cases.

The case-control study has been designed to select the NMS and NMS-like cases identified until december 31, 2020 by PCC-CNIT unit, while controls were selected among subjects who entered the hospital for a physical examination. The control group was composed by healthy individuals withouth a known history of drug-induced hyperthermia. Genetic variants of the *DRD2* gene (exposure variables) were genotyped by the researchers of the laboratory of Prof. Ornella Pastoris, Pharmacogenetics and Experimental Toxicology Laboratory, University of Pavia (https://dbb.unipv.it/eng/pharmacology-and-toxicology/) and Dr. Antonio Fiorenzo Peverali, Institute of Molecular Genetics "Luigi Luca Cavalli-Sforza", National Council of Research, Pavia (http://www.igm.cnr.it/).

Statistical analysis was carried out in collaboration with Proff. 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).

2.2 Recruitment of patients and description of the study

This study was approved by the Ethics Committee of IRCCS ICS Maugeri in Pavia, and informed consent was collected from each individual involved in this study.

The PCC-CNIT unit is an hospital-based unit (24/7) where medical clinical toxicologists advice on and assist for diagnosis and management of poisoning (mainly by telephone consultation) all the physicians requiring specialist consultation from the emergency department and intensive care units all over Italy.

Computerized medical records that contain detailed information on demographic data, history, clinical picture at admission and during hospital stay, treatments, clinical follow-up, and outcome, are registered in all the intoxication cases. A targeted follow-up is performed in all relevant cases to register clinical evolution and outcome.

Biological samples, i.e. blood, of the patients were collected upon informed consent (eventually obtained from the physician who was in charge of the patient, if the patient was unconscious). All samples were shipped to the PCC – CNIT unit at controlled temperature, collected and anonymized. A blood aliquot of each patient, included in the current study, was processed for the genetic analysis. Individuals without a known history of drug-induced hyperthermia were also enrolled in the present study as a control population. Upon informed consent, blood samples of these anonymized individuals were collected and processed as above.

So far, a cohort of 31 patients who experienced hyperthermia upon exposure to drugs or illicit substances were initially enrolled in the current study. According to causative agents, the patients were divided in two groups: the first group was composed by 12 patients who developed NMS following assumption of antypsichotics; the second group was composed by 13 patients who experienced NMS-like symptoms after exposure to illicit drugs, whereas 6 patients were excluded from the study because did not satisfy one or more inclusion criteria (see below).

2.3 Inclusion criteria of the study

NMS diagnosis was made based on the presence of all the following diagnostic criteria in each patients, developed on the basis of the guidelines

of the International Expert Consensus for NMS diagnosis (Gurrera *et al.*, 2011, 2017):

1. recent exposure to a dopamine antagonist;
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 the upper limit of normal [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 etiologies.

For patients presenting NMS-like symptoms following exposure to substances of abuse, diagnosis was based on the presence of all of the diagnostic criteria described above, except for the first one (recent exposure to dopamine antagonist).

For the control group, 69 individuals who entered the hospital for a physical examination were enrolled. The control groups was composed by individuals without a known history of drug-induced hyperthermia (20 males and 48 females; mean age 45.19 years, SD 14.11) .

All individuals (94) were European non-Finnish subjects.

2.4 Genetic analysis

2.4.1 Sample collection and DNA extraction

The blood specimens were collected from all patients and controls involved in our study. The peripheral whole blood samples were preserved in EDTA containing tubes at -20°C until analysis. Genomic DNA was extracted from whole blood by Blood & Tissue Genomic DNA Extraction kit (Fisher

Molecular Biology, Trevose, PA – USA). Each DNA extraction procedure was performed according to the protocol provided by the manufacturer (**Figure 12**):

1. Whole blood sample (200 µl) of was transferred from the EDTA containing tube to a microcentrifuge tube.
2. Proteinase K 10 mg/ml solution (20 µl) and of FSTG2 Buffer (200 µl) were added to the sample.
3. The sample was mixed thoroughly by pulse-vortexing and then lysed by incubation at 70 ºC for 10 minutes. During incubation, the sample was vortexed every 3-5 minutes.
4. Ethanol 96-100 % (200 µl) was added to the sample.
5. The sample was mixed thoroughly by pulse-vortexing for 30 seconds.
6. A FSTG column was place into a collection tube and the mixture was carefully transferred to the column.
7. The FSTG column containing the sample was centrifuged at $10{,}000 \times g$ for 1 minute and then placed to a new collection tube.
8. W1 Buffer (400 µl) was added to the FSTG column by centrifuge at $10{,}000 \times g$ for 1 minute, then the flow-through was discarded.
9. Wash Buffer (750 µl) was added to the FSTG column by centrifuge at $10{,}000 \times g$ for 1 minute, then the flow-through was discarded.
10. The sample was centrifuge for an additional 3 minutes to dry the column.
11. The FSTG column was placed to an elution tube.
12. Elution Buffer (100 µl) was dispensed onto the membrane centre of the FSTG column. When the elution solution was absorbed completely, about 3 minutes later, the FSTG column was centrifuged at $14{,}000 \times g$ for 1 minute to elute total DNA.
13. Step 12 was repeated.
14. The pure genomic DNA obtained (gDNA) was quantified by spectrophotometric analysis at 260 nm using a NanoDrop® ND 1000 instrument (NanoDrop,Thermo Fisher, MD, USA), and stored at -20 °C until use.

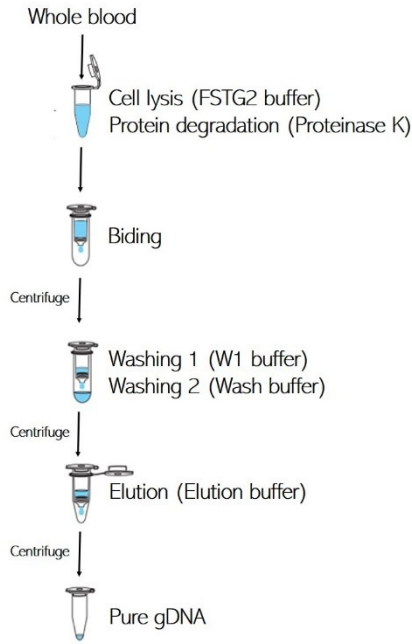

Figure 12. DNA extraction procedure. The picture shows a schematic representation of the gDNA extraction procedure from whole blood according to the protocol provided by the manufacturer of the DNA extraction kit.

2.4.2 PCR assays

Polymerase Chain Reaction (PCR) assays were carried out to amplify the target sequences on human genomic DNA extracted from the blood samples from the 94 individuals enrolled for this study. Primers were obtained by Eurofins Genomics (https://www.eurofinsgenomics.eu/). PCR assays were performed by GOTAQ Hot Start Green Master Mix G2 (Fisher Molecular Biology, Trevose, PA – USA) in a total volume of 35 µl. The reaction tubes were assembled on ice to avoid premature, nonspecific polymerase activity. Reactions were mixed thoroughly by pipetting or gentle vortexing followed by a brief spin in a microcentrifuge. Subsequently, the reaction tubes on ice were moved into a pre-warmed thermal cycler (iCycler™, Bio-Rad, Hercules, CA - USA). Oligonucleotide sequences and reaction conditions adopted are

reported in **Table 3**. After cycling, the reactions were stored at -20°C until genotyping.

Site	Oligonucleotide sequence (5'-3')	T_m	Reaction composition	PCR conditions	PCR product lenght (bp)	References
rs1800497	NMS1F: 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, 17.5 pmoles 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*	-
	NMS1R: ACGGCTCCTTGCCCTCTAG	61°				
rs1799978 rs1799732	677Fw: ACTGGCGAGCAGACGGTGAGGACCC	71.2°C	35 µl reaction mix containing 2-250 ng of genomic DNA, 0.4 X GOTAQ Hot Start Green Master Mix G2 and 12.5 pmoles of each primer, and nuclease-free water up to volume.	Initial denaturation at 98°C for 1 min, 35 cycles of denaturation at 98°C for 20 sec, annealing and extesion at 74°C for 5 min and final extension at 72°C for 10 min.	~303 *bp*	Arinami *et al.*, 1997
	676Rv: TGCGCGCGTGAGGCTGCCGGTTCGG	74.5°C				

Table 3. Oligonucleotide sequences and amplification conditions for the sites studied. The table shows the oligonucleotide sequences, the component volumes and the cycling condition applied to each PCR reaction to amplify the sites studied.

2.4.3 Genotyping

2.4.3.1 Sanger sequencing

PCR products were purified by Gel/PCR Extraction & PCR Clean Up kit (Fisher Molecular Biology, Trevose, PA - USA) according to the protocol provided by the manufacturer (**Figure 13**):

1. FSDF Buffer (5 volumes) was added to PCR product (20 µl). Then, the reaction was mixed well by vortexing.
2. A FSDF column was placed into a collection tube.
3. The sample mixture was transferred to the FSDF column and centrifuged at $11{,}000 \times g$ for 30 seconds, then the flow-through was discarded.
4. Wash Buffer (750 µl) was added to the FSDF Column and centrifuged at $11{,}000 \times g$ for 30 seconds, then the flow-through was discarded.
5. The sample mixture was centrifuged again at full speed ($\sim 18{,}000 \times g$) 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 dispensed onto the membrane centre of the FSDF column. When it was completely adsorbed by the membrane, about 1 minute later, the FSDS column was centrifuged at full speed ($\sim 18{,}000 \times g$) for 1 minute to elute the DNA.

Purified PCR products were sequenced by Sanger sequencing procedure (Eurofins Genomics, https://www.eurofinsgenomics.eu/).

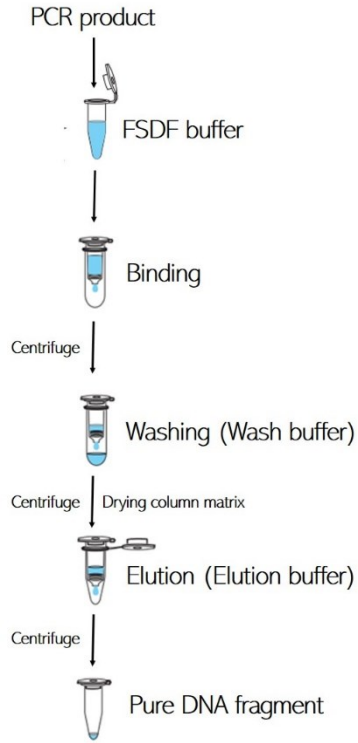

Figure 13. PCR product purification procedure. The picture shows a schematic representation of the PCR product purification procedure according to the protocol provided by the manufacturer of the DNA extraction kit.

2.4.3.2 Restriction fragment length polymorphisms analysis

Some PCR products were also subjected to restriction fragment length polymorphism (PCR-RFLP) analysis. The digestion with Taqα1 enzyme (NEB, New England Biolabs, Ipswich, MA – USA) of the 236 bp amplicon generated by PCR with NMS1F and NMS1R oligonucleotides was applied for the rs1800497 SNP. Conversely, the digestion of the 303 bp amplicon obtained by PCR with 677Fw and 676Rv oligonucleotides with BstNI (NEB, New England Biolabs, Ipswich, MA – USA) or MaeIII (Merck KGaA, Darmstadt, Germany) was carried out to investigate rs1799732 and rs1799978 variations, respectively. The procedures were performed according to the instructions provided by the manufacturers.

After the enzymatic digestion, restriction fragments were separated by 3% agarose/TBE 1X/ethidium bromide gel electrophoresis run at 80 volts for 60 – 75 minutes. Gels were imaged using a GelDoc imaging system with ethidium bromide filter (Bio-Rad Laboratories Inc, CA, USA). Fragments size was estimated by comparison with a 100 bp or 50 bp DNA ladder (Fisher Molecular Biology, Trevose, PA – USA).

2.5 Statistical analysis

Genotypes and allele frequencies of the analysed polymorphisms were inspected in NMS, NMS-like patients, healthy controls and the Gnom-AD database (https://gnomad.broadinstitute.org/). Allele frequencies in healthy controls were examined to detect any significant deviation from the Hardy–Weinberg Equilibrium using a goodness of fit χ^2 test.

Comparison of genotype distribution was evaluated in NMS vs control group and in NMS-like *vs* control group by means of Pearson Chi-square or Fisher exact tests. Specifically, *dominant genetic models* were used to make inference about genetic association of the specific risk allele with the outcome, considering subjects with at least one risk allele *vs* subjects without any risk allele. The risk allele was considered as the minor allele and the following categories, thus were compared: G/G + G/GG genotypes *versus*

GG/GG genotype for rs1799732; AA + AG *vs* GG for rs1800497 and CC + CT *vs* TT genotype for rs1799978.

Pearson's chi-squared test and Student's two-sample t-test with equal variances were applied to compare gender and age distributions, respectively, in the group of patients *vs* the control group.

Unconditional logistic regression analysis was performed to assess the sex and age adjusted estimate of NMS risk for subjects with G/G or G/GG rs1799732 genotypes with respect to subjects with G/G genotype. Adjusted Odds Ratios (OR) with 95% confidence intervals (95% CI) were derived and used as measure of effect.

A p-value less than 0,05 was considered as statistically significant and indicated by an asterisk (*). All the statistical analyses were performed using Stata 15 statistical software (Stata Corporation, College Station, TX, USA).

2.6 Bioinformatic analysis

Information about genes and gentic variants involved in this study were obtained from human genome assembly GRCh38.p13 by means of the genome browser Ensembl Release 101 (http://www.ensembl.org/index.html) and OMIM (https://www.omim.org/). Sources of genetic variants frequencies was gnomAD v2.1.1 (https://gnomAD.broadinstitute.org/). PROMO and AliBaba2.1 software were applied for a preliminary analysis of transcription factors (TF) consensus sites on the promoter region of *DRD2* gene (Messeguer *et al.*, 2002; Farré *et al.*, 2003; Matys *et al.*, 2003). Human Protein Atlas was source of RNA and protein expression data (https://www.proteinatlas.org/) (Uhlén *et al.*, 2015).

3 AIMS

MH, NMS, and SS the three main drug-induced conditions presenting with hyperthermia and muscle rigidity (Paden MS, Franjic L, 2013). These are complications related to the exposure to different substances, including halogenated anesthetic gasses and succinylcholine, antipsychotics and serotonergic agents, such as antidepressants and certain substances of abuse, respectively (Strawn, Keck and Caroff, 2007; Rosenberg *et al.*, 2015; Francescangeli *et al.*, 2019).

Although triggering mechanisms are different, they can present with similar clinical signs making differential diagnosis difficult, especially as regards NMS and SS, since patients are often treated with antipsychotics and antidepressants simultaneously (Rosenberg *et al.*, 2015; Tse *et al.*, 2015). Furthermore, clinical manifestations with SS and NMS overlapping features have been described also in association with certain substances of abuse, such as MDMA and cocaine (Daras *et al.*, 1995; Demirkiran, Jankovic and Dean, 1996; Wetli, Mash and Karch, 1996; Russell *et al.*, 2012).

Given similarities in clinical presentation, common underlying mechanism and shared genetic predisposition have been proposed (Daras *et al.*, 1995; Demirkiran, Jankovic and Dean, 1996; Gurrera, 2002; Kawanishi, 2003; Rusyniak and Sprague, 2005; Steele, Keltner and McGuiness, 2011; Ortiz *et al.*, 2020). Dopamine D_2 receptor is involved in thermoregulation, muscle tone, movement, behavior and cognitive function (Balthazar *et al.*, 2010; Beaulieu and Gainetdinov, 2011). Moreover, it is generally accepted that central dopaminergic blockade, especially of dopamine receptor D_2, may be a critical step for NMS onset. So far, studies about the involvement of genetic variants of the *DRD2* gene in NMS susceptibility have provided conflicting and unconclusive results (Kawanishi, 2003).

The present study was carried out on South European non-Finnish patients who developed hyperthermia and other clinical signs triggered by antipsychotics treatment or illicit drugs abuse. Genetic variants of the *DRD2* gene were investigated to discover putative associations to NMS or NMS-like diseases.

4 RESULTS

4.1 Inclusion Criteria and Patient Enrolment

4.1.1 Inclusion criteria

Over the duration of this three-year study, from 2017 to 2020, the Poison Control Center (PCC) and National Centre for Toxicological Information (CNIT) of IRCCS ICS Maugeri in Pavia was consulted by physician from the emergency departments and intensive care units all over Italy requiring advise for diagnosis and management of a total of 31 South European non-Finnish subjects presenting drug-induced hyperthermia.

On the basis of the guidelines of the International Expert Consensus for NMS diagnosis (Gurrera *et al.*, 2011, 2017), the following inclusion criteria were applied for the present study:

1. recent/current exposure to a trigger substance: A) dopamine antagonist; B) a substance of abuse, including novel psychoactive substances;
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 the upper limit of normal [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 etiologies.

4.1.2 Patients Enrolment

Among the 31 patients presenting drug-induced hyperthermia, one subject was diagnosed with MH following inhalation of halogenated anesthetic

gases; another subject was diagnosed with SS; a third patient was affected by Parkinson disease and developed hyperthermia without rigidity following abrupt withdrawl of dopaminergic therapy; a fourth subject was diagnosed with hyperthermia and muscle rigidity following long-acting injectable (LAI) paliperidone palmitate administration as a consequence of the accidental diffusion of a drug intended for intramuscular use into the blood vessel leading to high serum drug concentrations; finally two subjects showed no muscle rigidity.

Therefore, these 6 patients were excluded from the current study, since did not fullfil the above described inclusion criteria, while the remaining 25 patients (21 males and 4 females; mean age 39.92 years, SD 16.42) fullfilled the inclusion criteria of this study (**Figure 14**).

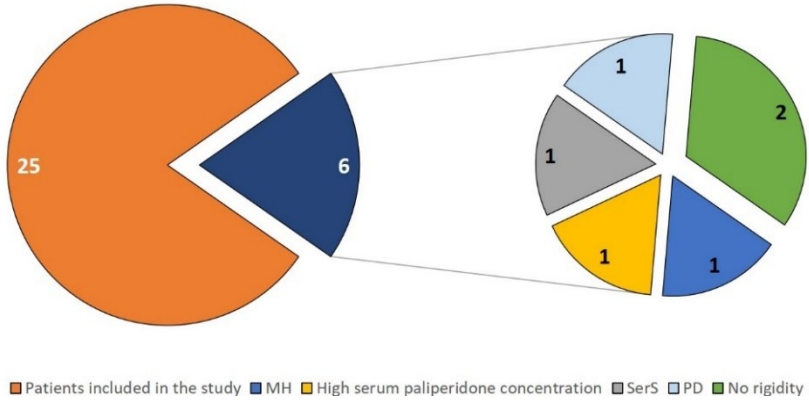

Figure 14. Inclusion and exclusion criteria. Pie chart describing the cohort of patients enrolled for the study. On the left is reported the number of patients included in the study (in orange). Patients excluded from the study (in blue) are reported in detail on the right.

Abbreviations: MH, Malignant Hyperthermia; PD, Parkinson disease; SS, serotonin syndrome.

Based on clinical signs and triggers, 12 patients were diagnosed with NMS, while NMS-like was described in 13 patients.

4.1.3 NMS Patients

Polypharmacy has been proposed as a risk factor for NMS, including both antipsychotic polypharmacy and/or their co-administration with other psychotropic drugs (Caroff and Mann, 1993; Nielsen *et al.*, 2012; Schneider *et al.*, 2020).

Among the 12 NMS patients investigated in this study, 4 patients were treated with an antipsychotic monotherapy, of which 1 patient received the FGA haloperidol, 2 patients received the SGA aripiprazole, and 1 patient developed recurrent NMS episodes upon treatments with four consecutive different antipsychotic monotherapies. The remaining 8 patients experienced NMS upon the administration of multiple medications, of which 3 patients were treated with a cocktail of FGA and SGA antipsychotics, whereas 5 patients were treated with a cocktail containing antipsychotics and other psychotropic drugs, such as antidepressants, mood stabilizers, anti-parkinsonians, benzodiazepines, and memantine.

SGAs were the most recurrent drugs, being involved in 11 out 12 NMS cases. The SGA aripiprazole was the trigger in 6 cases, whereas the FGA haloperidol was the second most recurrent antipsychotic observed in this study cohort, being involved in 5 cases.

Another recurrent risk factor was the variation of the treatment plan. Indeed, NMS onset was observed in 5 patients within 30 days after the change of the treatment plan. Moreover, 1 patient developed NMS upon the first antipsychotic assumption, confirming that the initial phase or variation of the treatments are risk factors.

According to other risk factors described, we also observed NMS onset concurrent to high ambient temperature (4 cases) and comorbidities (2 cases).

Overall, 11 out 12 patients who developed NMS had at least one risk factor concurrent to antipsychotic therapy, while 1 subject developed NMS upon antipsychotic monotherapy only (**Figure 15**).

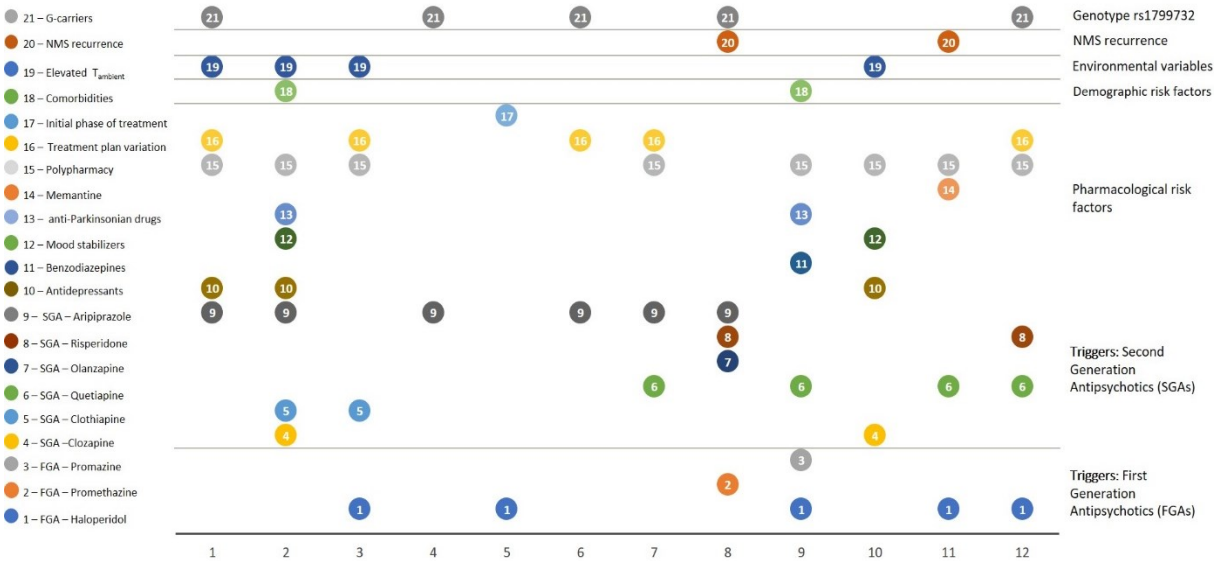

Figure 15. Triggers and risk factors among NMS patients. Diagram summarizing triggers and risk factors, as reported on the right, observed in our cohort of NMS patients. Each trigger and risk factor has been assigned a numbered and coloured dot as specified on the left. On the abscissa axis NMS patients from 1 to 12 are reported.

4.1.4 NMS-like Patients

Within the group of patients who developed NMS-like symptoms following exposure to substances of abuse, cocaine played a major role, being involved in 7 out of 13 cases. Among these, 2 cases concerned only cocaine abuse, while the other 5 involved the use of other substances in addition to cocaine, such as MDMA (1 case), cannabinoids (2 cases), and atropine (2 cases). MDMA, or similar compounds MDEA and MDA, were involved in 4 out of 13 cases, 1 of which in association with cocaine. Other substances detected in sporadic cases were the MDMA derivatives 6-(2-amminopropil)benzofuran (6-APB), and paramethoxymethamphetamine (PMMA), that was found in association to MDMA in 1 case, and the ketamine derivative methoxetamine (MXE) (**Figure 16**).

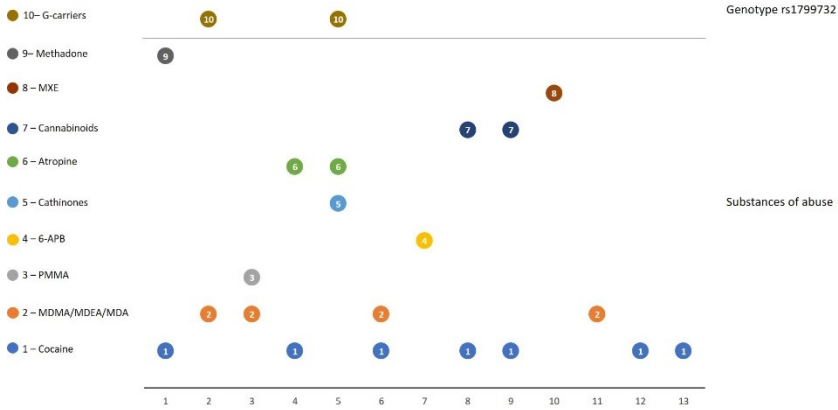

Figure 16. Triggers among NMS-like patients. Diagram summarizing triggers observed in our cohort of NMS-like patients. Each trigger has been assigned a numbered and coloured dot as specified on the left. On the abscissa axis NMS-like patients from 1 to 13 are reported. Cocaine was the most frequent substance of abuse among patient s who developed NMS-like symptoms, followed by MDMA and related substances.

4.1.5 Enrolment of Control Individuals

As control group, 69 European non-Finnish subjects who entered the hospital for a physical examination were enrolled. The control group was

composed by 69 individuals (20 males and 48 females; mean age 45.19 years, SD 14.11) without a known history of drug-induced hyperthermia.

To check for potential biases due to enrolment processes, i.e. founder effect or genetic isolation, the frequency distribution of the three genetic variants, rs1800497, rs1799732 and rs1799978, investigated in this project was firstly evaluated among the control individuals and compared with the values reported by the gnomAD database for the European population (https://gnomAD.broadinstitute.org/). The allelic distribution among the controls was in the range reported by the gnomAD database for Southern Europe, in agreement with the ethnic distribution of the population residing in this geographical area and did not present any significant deviation from the Hardy–Weinberg Equilibrium for rs1800497 and rs1799732 ($p>0.05$), while it deviated slightly for rs1799978 ($p=0.04$) (**Table 4**, **Figure 17**).

rs1800497				
	Controls ($n = 69$)		gnomAD ($n = 5665$)	
	N	frequency	N	frequency
allele G	118	0.855	9450	0.834
allele A	20	0.145	1880	0.166
rs1799978				
	Controls ($n = 69$)		gnomAD ($n = 53$)	
	N	frequency	N	frequency
allele T	131	0.949	100	0.943
allele C	7	0.051	6	0.057
rs1799732				
	Controls ($n = 69$)		gnomAD ($n = 46$)	
	N	frequency	N	frequency
allele GG	128	0.928	85	0.924
allele G	10	0.724	7	0.076

Table 4. Allelic distributions of the variants studied among controls in comparison to gnomAD database. The allelic distribution of the variants studied among controls is reported in comparison to allelic frequencies reported by gnomAD database (v2.1.1) for the Southern European population.

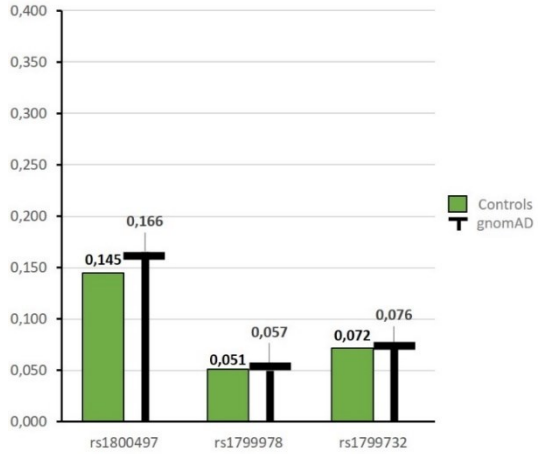

Figure 17. Bar plot of minor allele frequencies of the variants investigated. The distribution of the minor allele of each of the three variants investigated is reported comparing frequencies observed among controls (in green) to data reported by gnomAD database (v2.1.1) for the Southern European population (in black).

4.1.6 Gender and age distribution

Next, gender and age distribution were investigated among the patients and control individuals. The group of patients was composed by a total of 25 subjects, of which 21 males and 4 females (mean age 39.92 years, SD 16.42). The control group was composed by 69 generally healthy individuals, of which 20 males and 48 females (mean age 45.19 years, SD 14.11). For one of the individuals enrolled in the control group, no information regarding gender or age was available.

Gender distribution between the group of patients and the group of controls are shown in (**Figure 19**). A significantly unbalanced composition was found for gender between patients and controls on a Pearson's chi-squared test ($X_1^2 = 22.0975, p = 0.000$). The possible interference of gender was subsequently evaluated in the statistical analysis (see later).

The age distribution showed no significant distributional difference at diagnosis (years) between the patients with drug-induced hyperthermia and the controls by a Student's two-sample *t*-test with equal variances ($t_{91} = 1.5275, p = 0.0651$) (**Figure 18**).

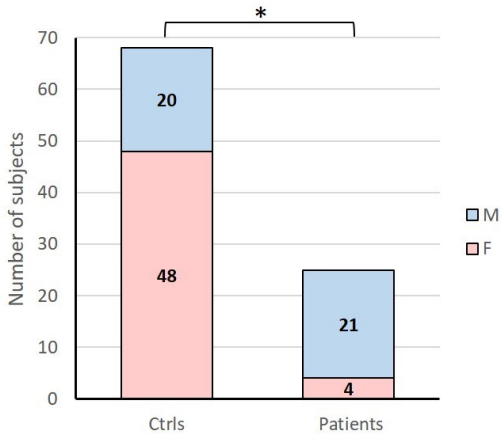

Figure 19. Gender distribution between patients and controls. Bar plot showing the distribution of men (in light blue) and women (in pink) between controls and patients. Pearson's chi-squared test significance is reported with an asterisk ($^*p < 0.05$).

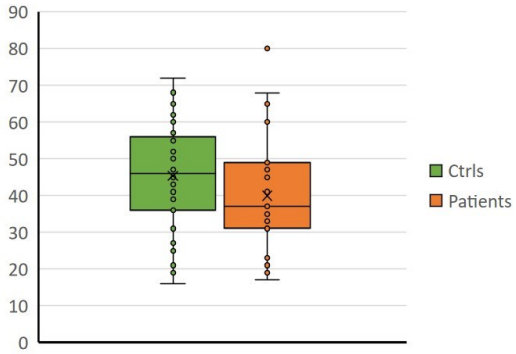

Figure 18. Age distribution between patients and controls. Box-and-whisker plot showing the distribution for age at diagnosis (years) between the patients with drug-induced hyperthermia (in orange) and the controls (in green) is shown. Whiskers indicate variability outside the upper and lower quartile. Outliers are reported as individual dots. The average value is indicated by an x. The distributional difference for age was not significant on Student's t-test ($p > 0.05$).

4.2 Genetic analysis

Reduced levels of dopamine metabolite in the 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 central dopaminergic blockade, especially of dopamine receptor D_2, plays a critical role for the onset of NMS, however biochemical mechanisms underlying NMS are still poorly understood (Nisijima and Ishiguro, 1995; Jauss *et al.*, 1996; Strawn, Keck and Caroff, 2007; Berman, 2011). In addition, virtually all antipsychotics act as antagonists at dopamine D_2 receptor, thus they have been hypothesised as trigger of the NMS; events indistinguishable from NMS have been occasionally described also following other pharmacological interventions leading to a reduction in dopaminergic neurotransmission, such as exposure of DA-antagonists other than antipsychotics, and sudden withdrawal of dopamine agonists in patients with Parkinson's disease (Caroff and Mann, 1993; Strawn, Keck and Caroff, 2007). Finally, dopaminergic drugs have been successfully used in NMS treatment (Pileggi and Cook, 2016). Thus, many studies about genetic predisposition to NMS have focused on dopamine receptor D_2 gene, although resulting in mixed and conflicting results (Kawanishi, 2003).

Human *DRD2* gene (ENSG00000149295) codes the D_2 receptor and it maps on Chromosome 11: 113,409,605-113,475,691 reverse strand (**Figure 20**).

So far, 296 orthologues and 19 paralogues have been described: https://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000149295;r=11:113409605-113475691 . This gene codes 10 transcripts generated by alternative splicing, 6 of which are protein coding. Among these, the canonical transcripts of 2808 bp (ENST00000362072.8) has 8 exons of which 7 exons codes the 443 aminoacidic residues of the D_2 long isoform of the receptor that is expressed mainly in post-synaptic neurons. Moreover, in the 2433 bp transcript, ENST00000346454.7, the exon 6 is skipped, thus the remaining 6 exons code the 414 residues of the D_2 short isoform that is expressed mainly in post-synaptic neurons (Khan *et al.*, 1998).

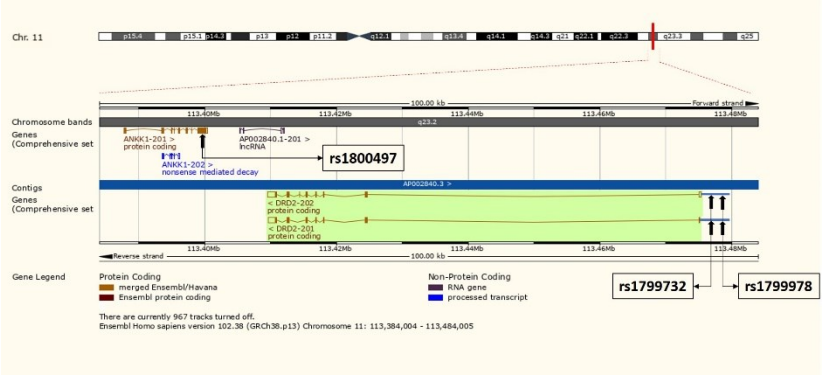

Figure 20. Human DRD2 genomic context. At the top, the schematic representation of human chromosome 11 is reported. The cytogenetic position of *DRD2* gene is indicated by a red bar. The detail of the corresponding genomic region from Ensembl *Homo sapiens* version 102.38 (GRCh38.p13) is reported at 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. Introns are indicated by lines connecting boxes. The genetic variants investigated in this project, rs1800497, located within exon 8 of *ANKK1* gene, rs1799732 and rs179978, located within promoter region of *DRD2* gene, are boxed and their position is indicated by arrows.

In the present study, three SNPs were mainly investigated on the cohort of NMS and NMS-like patients and control individuals. The rs1800497 SNP mapped about 10kb at the 5' side of the *DRD2* gene into the exon 8 of the adjacent *ANKK1* gene that encodes for ankyrin repeat and protein kinase domain-containing protein 1. Given that *DRD2* gene is mapped on the reverse strand of the Human Genome Reference Consortium (GRCh38.p13), this SNP has been previously investigated as a putative variation affecting the *DRD2* gene regulation downstream of the transcribed strand (Noble, Blum and Khalsa, 1991; Thompson *et al.*, 1997; Pohjalainen *et al.*, 1998; Neville, Johnstone and Walton, 2004; Hirvonen, Laakso, *et al.*, 2009; Hirvonen, Lumme, *et al.*, 2009b). Conversely, the rs1799732 SNP was mapped on the 3' side of the *DRD2* gene according to the Reference genome (GRCh38.p13), however this SNP is actually mapped into the promoter of the human *DRD2* gene at about 132bp from the Transcription Start Site (TSS) (Arinami *et al.*, 1997). Notably, these two SNPs had been previously studied in association to NMS susceptibility in the Japanese population with mixed and inconclusive

results (Kawanishi, 2003). A third genetic variant, namely rs1799978, also located at the 3' side of the *DRD2* gene, was studied. According to the reverse strand of the GRGh38.p13, this SNP has been mapped within the promoter region of *DRD2* gene about 232 bp upstream of the TSS of the *DRD2* gene. This SNP was previously investigated for putative association to antipsychotic treatment response (Arinami *et al.*, 1997; Xing *et al.*, 2007; Zhang, Lencz and Malhotra, 2010).

4.2.1 The Taq1A variant rs1800497 in the *ANKK1* locus

The variant rs1800497, also named Taq1A or TaqIA, maps at position 11:113400106 into exon 8 of the *ANKK1* gene, located about 10kb at the 5' side of the *DRD2* gene. It is characterized by a G to A missense change (forward strand, Ensembl Release 101, 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

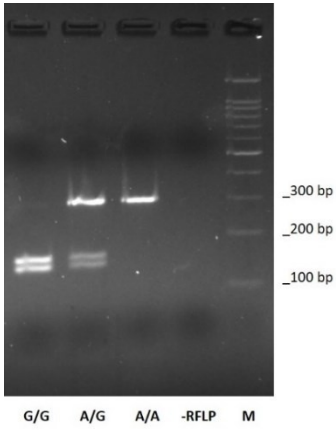

Figure 21. PCR-RFLP for rs1800497 variant within exon 8 of the human *ANKK1* gene. The 236 bp fragments amplified by PCR with primers NMS1F and NMS1R were digested with TaqαI enzyme. The sizes of the digestion fragments resolved on a 3% agarose-TBE gel for rs1800497 genotypes were: A/A 236 bp (uncleaved); A/G 236, 125, 111 bp; G/G 125, 111 bp. Lane M represents a 100 bp DNA ladder.

Sanger sequencing were applied to investigate this SNP on a total of 94 individuals (25 patients and 69 controls) (**Figure 21**).

Among the 69 controls, 2 individuals had the genotype A/A, 16 had the genotype A/G and 51 individuals had the genotype G/G, corresponding to a frequency of 0.145 for allele A and 0.855 for allele G. Accordingly, the A allelic frequency is in the expected range as in GnomAD database. In contrast, a total of 16 patients had the G/G genotype whereas 8 had the genotype A/G and 1 had the genotype A/A, corresponding to a frequency of 0.200 for allele A and 0.800 for allele G. Therefore, a slight increase of A-allele frequency was observed among the patients in comparison to controls. However, both values appear in the expected range for the Southern European population according to GnomAD database (**Figure 22**).

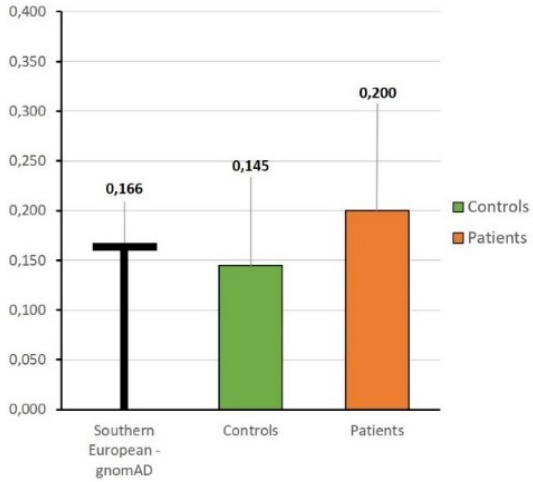

Figure 22. Minor allele frequency of rs1800497 variant of *ANKK1* gene in the study cohort. The bar plot shows the frequency distribution of the minor allele A of the variant rs1800497 between patients (in orange) and controls (in green). The frequency of allele A for the Southern European population according to gnomAD database (v2.1.1) is also reported (in black).

A dominant genetic model was used to make inference about genetic association of the specific risk allele A with the outcome, considering subjects with at least one risk allele *vs* subjects without any risk allele. Thus, for rs1800497 AA + AG genotypes *vs* GG genotype were compared. However, no significant variation of A-carrier genotype (A/G + A/A genotypes) allele frequency was found between patients and controls ($p = 0.350$) (**Table 5**).

rs1800497					
	Patients ($n = 25$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
G/G	16	0.640	51	0.739	
A/G	8	0.320	16	0.232	
A/A	1	0.040	2	0.029	
A/A + A/G	9	0.360	18	0.261	0.350

Table 5. Genotypes distribution of the rs1800497 variant of *ANKK1* gene in the study cohort. Genotypes distribution of the rs1800497 variant observed between patients and controls is reported. The genetic association was tested considering A/A + A/G *vs* G/G genotypes.

No significant variation of A-carrier genotype (A/G + A/A genotypes) frequency was also observed even by comparing each patient cohorts with control individuals (**Table 6**, **Table 7**, **Figure 23**).

rs1800497					
	NMS patients ($n = 12$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
G/G	8	0.667	51	0.739	
A/G	3	0.250	16	0.232	
A/A	1	0.083	2	0.029	
A/G + A/A	4	0.333	18	0.261	0.602

Table 6. Genotypes distribution of the rs1800497 variant between NMS patients and controls. The genotypes distribution of the rs1800497 variant observed between NMS patients and controls is shown. The genetic association was tested considering A/A + A/G vs G/G genotypes.

rs1800497					
	NMS-like patients ($n = 13$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
G/G	8	0.615	51	0.739	
A/G	5	0.385	16	0.232	
A/A	0	0.000	2	0.029	
A/G + A/A	5	0.385	18	0.261	0.362

Table 7. Genotypes distribution of the rs1800497 variant between NMS-like patients and controls. The genotypes distribution of the rs1800497 variant observed between NMS-like patients and controls is shown. The genetic association was tested considering A/A + A/G vs G/G genotypes.

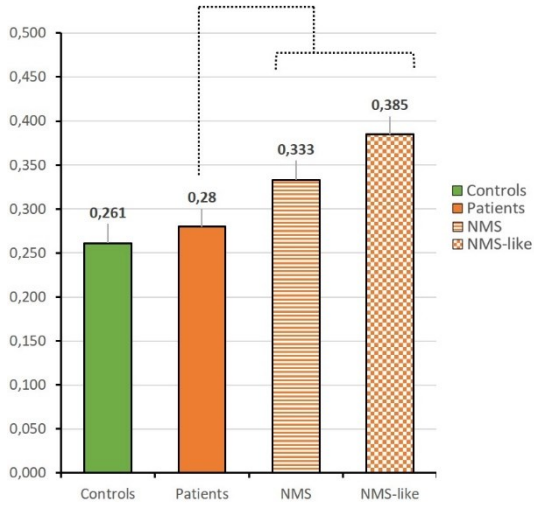

Figure 23. A-carrier genotype frequency of rs1800497 between NMS and NMS – like patients. The bar plot shows the frequency distribution of the genotypes carrying the risk allele A of the variant rs1800497 (G/A + A/A genotypes) between NMS (orange lined pattern) and NMS-like patients (orange squared pattern). Controls and overall patient are shown by green and orange filled bars. No significant variation of A-carrier genotype (A/G + A/A genotypes) frequency was observed (p>0.05).

4.2.2 The *DRD2* promoter variant rs1799978

The variant rs1799978 is located at the 3' side of the *DRD2* gene at position chromosome 11:113475629 (forward strand, Ensembl Release 101, GRCh38.p13). On the reverse strand it is mapped in the *DRD2* promoter at 231 bp upstream of the TSS (https://www.ensembl.org/Homo_sapiens/Variation/Explore?db=core;r=11:113475129-113476129;v=rs1799978;vdb=variation;vf=84078906) (**Figure 24**).

Figure 24. Nucleotide sequence of the promoter region and exon 1 of the *DRD2* gene amplified by PCR. The nucleotide sequence of the portion of the promoter region and exon 1 of the *DRD2* gene amplified by PCR is shown. Primers used for PCR are reported by arrows. The target sequence of 303 bp amplified by PCR is highlighted by a black line. The location of the rs1799732 variant and the rs1799978 variant are indicated by a red and blue box, respectively.

Abbreviations: TSS, transcription start site.

It consists of a nucleotide change from T to C (forward strand) of the Reference Genomic sequence (Enembl Release 101, GRCh38.p13: T → C). It was historically named A-241G, since it referred to the nucleotide change from A to G of the reverse strand and investigated as a RFLP with MaeIII restriction enzyme (Arinami *et al.*, 1997). The C allele is the MAF (minor allele frequency) with a frequency of 0.12, and the highest MAF observed in any population is 0.28 observed in the Gambian in Western Division in

Gambia. According to gnomAD genomes v2.1.1 the C allele frequency in the non-Finnish European population (NFE) is 0.055.

PCR with *D2-677Fw* and *D2-676Rv* primers was carried out to amplify a 303 bp amplicon on human genomic DNA. PCR products were then analysed by MaeIII RFLP analysis and Sanger sequencing on a total of 94 individuals (25 patients and 69 controls) (**Figure 25**).

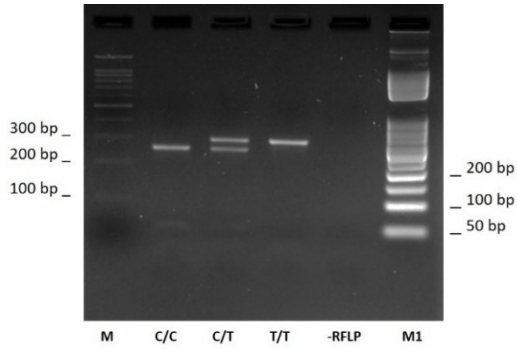

Figure 25. PCR-RFLP for rs1799978 variant in the promoter region of the human *DRD2* gene. The 303 bp fragments amplified by PCR with primers D2-677Fw and D2-676Rv were digested with MaeIII enzyme. The 303bp amplicon remained uncleaved when the reference allele T was present, whereas it was cut in 260 bp and 43 bp fragments in presence of the he alternative allele C (forward strand). Thus, the sizes of the digestion fragments resolved on a 3% agarose-TBE gel for rs1799732 genotypes were: T/T 303 bp (uncleaved); C/T 303, 260, 43 bp; C/C 260, 43 bp. Lane M and lane M1 represent a 100 bp and a 50 bp DNA ladder, respectively.

Among the 69 control individuals, 63 had the T/T genotype, 5 the C/T genotype and 1 C/C genotype, respectively. Thus, the allelic frequencies calculated for control individuals enrolled in this study were 0.949 for the allele T and 0.051 for the variant allele C, consistently to the C allelic frequency expected for the Southern European Population according to in gnomAD database. Among the 25 patients, 24 had the T/T genotype and 1 the C/T genotype, corresponding to an allelic frequency of 0.980 for the allele T and 0.020 for the variant allele C. Therefore, a slight decrease of C-allele frequency was observed among the patients in comparison to controls, nevertheless these values appear similar to those reported for the Southern European population by GnomAD database (**Figure 26**).

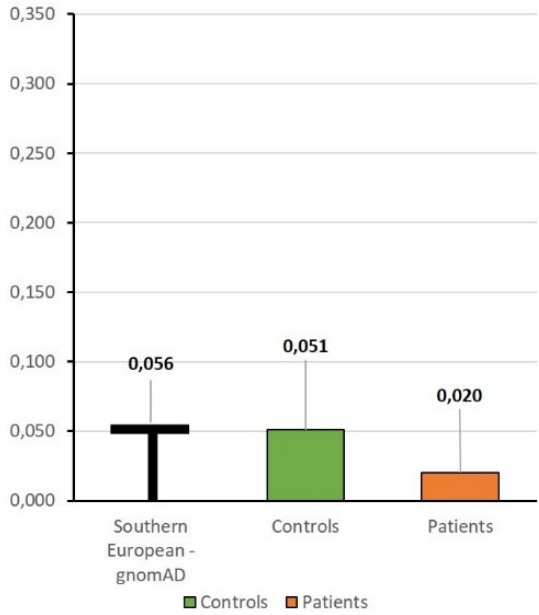

Figure 26. Minor allele frequency of rs1799978 variant of *DRD2* gene in the study cohort. The bar plot shows the frequency distribution of the minor allele C of the variant rs1799732 between patients (in orange) and controls (in green). The frequency of allele C for the Southern European population according to gnomAD database (v2.1.1) is also reported (in black).

Dominant genetic model was used to make inference about genetic association of the specific risk allele variant with the outcome. As regards rs1799978, minor allele C was considered as risk allele. Thus, comparisons were made between subjects with at least one risk allele (CC + CT genotypes) *vs* subjects without any risk allele (TT genotype). However, no significant variation of C-carrier genotype (C/C + C/T genotypes) frequency was found between patients and controls ($p = 0.670$) (**Table 8**) .

rs1799978					
	Patients ($n = 25$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
T/T	24	0.960	63	0.910	
C/T	1	0.04	5	0.072	
C/C	0	0.000	1	0.014	
C/C + C/T	1	0.04	6	0.087	0.670

Table 8. Genotypes distribution of the rs1799978 variant of *DRD2* gene in the study cohort. The genotypes distribution of the rs1799978 variant observed between patients and controls is reported. The genetic association was tested considering C/C + C/T *vs* T/T genotypes.

Moreover, no significant variation of C-carrier genotype (C/C + C/T genotypes) frequency was also observed even by comparing each patient cohorts with control individuals (**Table 9**, **Table 10**, and **Figure 27**).

rs1799978					
	NMS patients ($n = 12$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
T/T	12	1	63	0.910	
C/T	0	0.000	5	0.072	
C/C	0	0.000	1	0.014	
C/C + C/T	0	0.000	6	0.087	ND

Table 9. Genotypes distribution of the rs1799978 variant between NMS patients and controls. The genotypes distribution of the rs179978 variant observed between NMS patients and controls is shown. The genetic association was not tested (ND, not detected), lacking the C/C + C/T genotypes.

rs1799978					
	NMS- like patients ($n = 13$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
T/T	12	0.923	63	0.910	
C/T	1	0.077	5	0.072	
C/C	0	0.000	1	0.014	
C/C + C/T	1	0.077	6	0.087	0.905

Table 10. Genotypes distribution of the rs1799978 variant between NMS-like patients and controls. The genotypes distribution of the rs1799978 variant observed between NMS-like patients and controls is shown. The genetic association was tested considering C/C + C/T *vs* T/T genotypes.

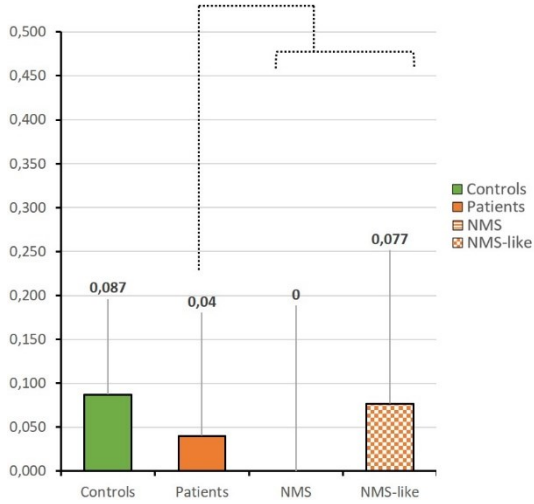

Figure 27. C-carrier genotype frequency of rs1799978 between NMS and NMS – like patients. The bar plot shows the frequency distribution of the genotypes carrying the risk allele C of the variant rs1799978 (C/C + C/T genotypes) between NMS (orange lined pattern) and NMS-like patients (orange squared pattern). No NMS patient carrying C/C or C/T genotype was found. Controls and overall patient are shown by green and orange filled bars. No significant variation of C-carrier genotype (C/C + C/T genotypes) frequency was observed ($p > 0.05$).

4.2.3 The *DRD2* promoter variant rs1799732

Accordig to the reference sequence of the human genome, the variant rs1799732 is mapped at the 3' side of the *DRD2* gene and it is classified as INDEL, indeed the reference allele G at position Chr 11: 113475529 bp, is changed to GG allele by an insertion of G nucleotide (forward strand, Ensembl Release 101, GRCh38.p13). The flanking sequence is: TCCTCGGCGATCCCCGGCCT**[G/GG]**GAACGGGTAGGAGGGGTTGG. This variant was historically named -141C ins/del, since it resulted from a C insertion on the promoter sequence of *DRD2* gene that maps on the reverse strand (https://www.ensembl.org/Homo_sapiens/Variation/Explore?r=11:113475030-113476030;v=rs1799732;vdb=variation;vf=84078598) of the human reference genome sequence. However, the most recent revision of the human reference sequence (GRCh38.p13) maps this variant at 131bp upstream of the TSS of the *DRD2*

gene. Although the reference allele G is also the ancestral allele, it is less frequent than the GG allele. The minor allele frequency (MAF) of the G allele as observed in 1000 Genomes Phase 3 combined population is 0.24 (G), whereas the highest MAF observed in any population is 0.48 and it refers to the African Ancestry population in the Southwestern US. According to gnomAD genomes v2.1.1, the G allele frequency in the non-Finnish European population (NFE) is 0.097, whereas the frequency of the GG allele is 0.903.

The promoter region of the *DRD2* gene was amplified with the primer set *D2-677Fw* and *D2-676Rv* to generate a 303bp fragment on gDNA templates by employing genomic DNA templates purified from 94 individuals enrolled in this study . PCR products were sequenced by Sanger procedure and the rs1799732 SNP was also investigated by RFLP analysis with BstNI enzyme. Restriction analysis of the 303bp amplicon generated the 159 bp and 144 bp fragments on the amplicon with the GG allele (forward strand), whereas the amplicon containing the G reference allele remained uncleaved. Thus, the rs1799732 RFLP pattern with BstNI enzyme was resolved on a 3% agarose-TBE gel and visualized under UV-light after ethidium bromide staining. The expected size of the digested band corresponding to the following genotypes were: G/G 303 bp (uncleaved); G/GG 303bp, 159bp, 144bp; GG/GG 159bp, 144bp (**Figure 28**).

Thus, the genotypic frequencies for control individuals were 0.869 for the GG/GG genotype, 0.116 for G/GG genotype, and 0.014 for G/G genotype. Based on these data, the allelic frequencies calculated for control individuals enrolled in this study were 0.928 for the variant allele GG, and 0.072 for the reference allele G. These values appear slightly lower from those reported for NFE super-population by the gnomAD database (see above). The allele frequencies of control individuals are, however, the same as those reported by the gnomAD database for the Southern European population (G: 0.924 and

GG: 0.076), in accordance with the geographical context where the subjects who participated in the study had been recruited.

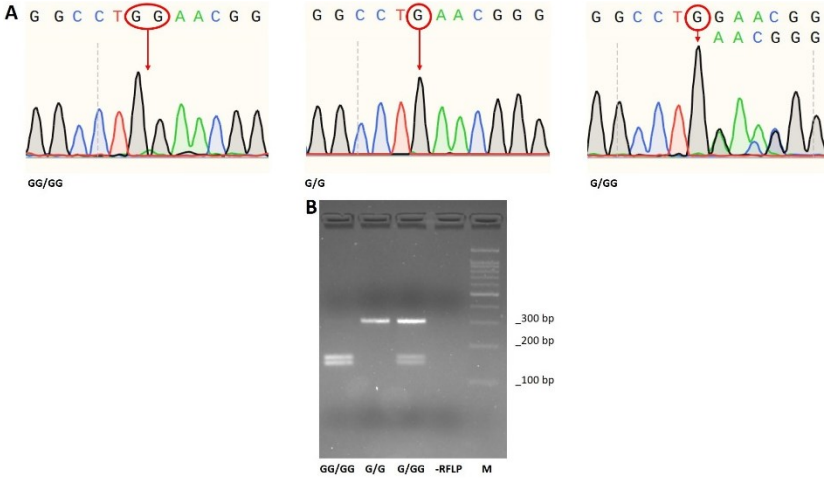

Figure 28. Genotyping for rs1799732 variant in the promoter region of the human *DRD2*. A Sanger's sequencing. Electropherograms show the homozygous GG/GG (left), the homozygous G/G (centre), and the heterozygous G/GG (right) genotypes. **B** PCR-RFLP analysis. The 303 bp fragments amplified by PCR with 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: G/G 303 bp (uncleaved); G/GG 303, 159, 144 bp; GG/GG 159, 144 bp. Lane M represents 100 bp DNA ladder.

The analysis of this variant on all 25 patients, revealed 18 patients with GG/GG genotype, and 7 patients with G/GG genotype. No patient carrying G/G genotype was found. Thus, genotype frequencies for patients were 0.72 for the GG/GG genotype and 0.28 for the G/GG genotype. Given the small number of G/G subjects, for the statistical analysis we classified subjects into G-carriers (pool of G/G and G/GG genotypes) and G non-carriers (GG/GG genotype only). Although the frequency of the G-carriers among patients raised about 2 fold over control individuals (0.28 *vs* 0.13, respectively), the difference was not significant on Fisher's exact test ($p = 0.085$) (**Table 11** and **Figure 29**).

rs1799732					
	Patients ($n = 25$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
GG/GG	18	0.720	60	0.870	
G/GG	7	0.280	8	0.116	
G/G	0	0.000	1	0.014	
G/G + G/GG	7	0.280	9	0.130	0.085

Table 11. Distribution of the rs1799732 variant of *DRD2* gene in the study cohort. The genotypic distribution of rs1799732 variant of *DRD2* between patients and controls is shown. The genetic association was tested considering G/G + G/GG genotypes *vs* GG/GG genotypes.

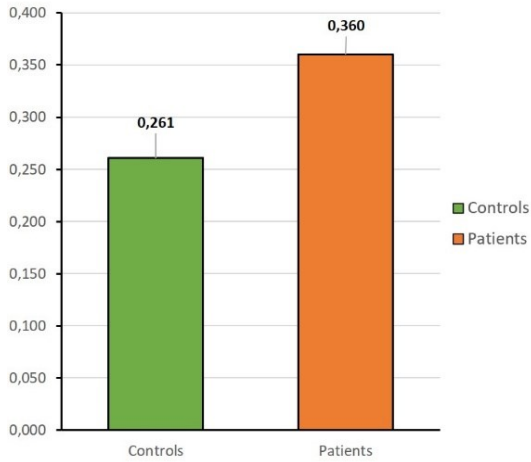

Figure 29. Frequency distribution of G-carriers between patients and controls. The bar plot shows the frequency of G-carrier subjects (G/GG + G/G genotypes) between patients (in orange) and controls (in green). The difference was not significant on Fisher's exact test ($p > 0.05$).

Next, the statistical analysis was performed on each group of patients that were grouped in NMS and NMS-like patients according to clinical signs and triggers, such as antipsychotic drugs vs illicit substances, respectively.

Among the NMS patients, 5 patients out of 12 had the G/GG genotype (G-carrier individuals), while 7 NMS patients had the GG/GG genotype (G-non carrier individuals). Upon statistical analysis, this resulted in a G-carriers frequency of 0.417 that is a statistically significant increase of about 3.2-fold over controls on 1-sided Fisher's exact test ($p = 0.029$) (**Table 12**, **Figure 30**).

rs1799732					
	NMS patients ($n = 12$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
GG/GG	7	0.583	60	0.870	
G/GG	5	0.417	8	0.116	
G/G	0	0.000	1	0.014	
G/G + G/GG	5	0.417	9	0.130	*0.029

Table 12. Distribution of the rs1799732 variant of *DRD2* gene in the study cohort. The genotypic distribution of rs1799732 variant of *DRD2* among NMS patients and controls is shown. The genetic association was tested considering G/G + G/GG genotypes *vs* GG/GG genotypes.

Contrary, only 2 out of 13 patients who developed NMS-like symptoms upon abuse of substances carried the reference allele G, resulting in a G-carrier frequency of 0.154 that was not significantly different from the control population on 1-sided Fisher's exact test ($p = 0.554$) (**Table 13**, **Figure 30**).

rs1799732					
	NMS-like patients ($n = 13$)		Controls ($n = 69$)		$p - value$
	N	frequency	N	frequency	
GG/GG	11	0.846	60	0.870	
G/GG	2	0.154	8	0.116	
G/G	0	0.000	1	0.014	
G/G + G/GG	2	0.154	9	0.130	0.554

Table 13. Distribution of the rs1799732 variant of DRD2 gene between NMS-like patients and controls. The genotypic distribution of rs1799732 variant between NMS-like patients and controls is shown. The genetic association was tested considering G/G + G/GG genotypes vs GG/GG genotypes.

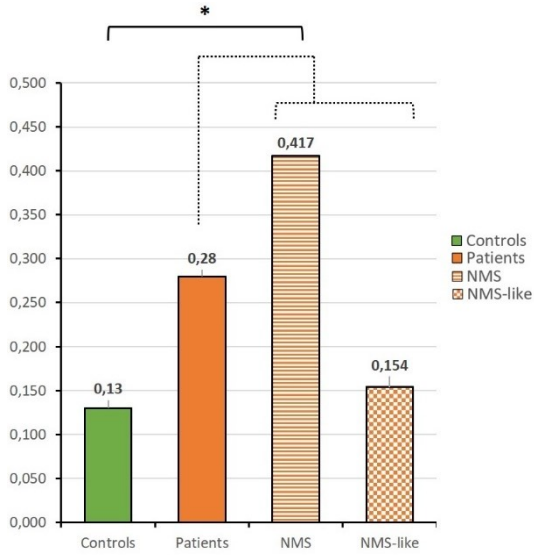

Figure 30. Frequency distribution of G-carriers between NMS and NMS-like patients. The bar plot shows the frequency distribution of the G-carriers (G/G + G/GG genotypes) of the variant rs1799732 between NMS (orange lined pattern) and NMS-like patients (orange squared pattern). Controls and overall patients are shown by green and orange filled bars. The distributional difference of the G-carrier subjects between controls and NMS patients was statistically significant on Fisher's exact test ($*p < 0.05$).

To check for potential biases due to unbalanced gender composition of the sample, gender distribution according to genotypes was also considered. Among a total of 16 G-carriers, 9 (0.563) were females and 7 were males (0.437); whereas 43 (0.558) females and 34 (0.442) males were G-non carriers individuals. No significant distributional difference was found for gender between G-carrier individuals and G-non carrier individuals on a Pearson's chi-squared test ($X_1^2 = 0.0009, p = 0.976$). Therefore, gender is not a confounding element or an effect modifier of the relationship between the disease and the genetic risk factor: because it is differentially distributed among cases and controls but it is not associated with the variant (**Table 14**, **Figure 31**).

rs1799732					
	F		M		$p-value$
	N	frequency	N	frequency	
GG/GG	43	0.558	34	0.442	
G/G + G/GG	9	0.559	7	0.441	0.976

Table 14. Gender distribution according to genotypes. Gender distribution according to genotypes is shown. The association was tested considering G/G + G/GG genotypes *vs* GG/GG genotypes.

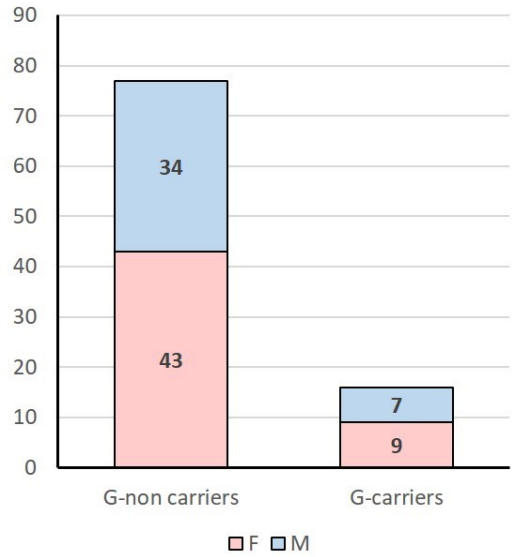

Figure 31. Bar plot of gender distribution according to genotypes. Distribution of males (in light blue) and females (in pink) according to G-carrier (G/GG + G/G) and G-non carriers (GG/GG) genotypes is shown by a bar plot. No significant distributional difference was found on a Pearson's chi-squared test ($p > 0.05$).

No significant difference was found for age at diagnosis (years) between the patients with drug-induced hyperthermia and the controls on a Student's two-sample *t*-test with equal variances ($t_{91} = 1.5275, p = 0.0651$)

(**Figure 18**). Moreover, no difference was found for age between G-non carrier individuals (mean age 44.35 years, SD 14.63) and G- carrier individuals (mean age 41 years, SD 32.42) on a Student's two-sample *t*-test with equal variances ($t_{91} = 0.8192, p = 0.2074$) (**Figure 32**).

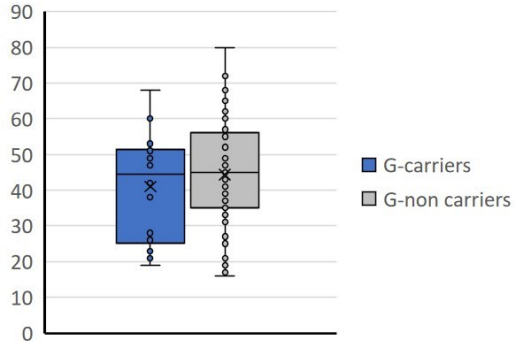

Figure 32. Age distribution according to genotypes. Box-and-whisker plot showing the distribution for age according to G-carrier (G/GG + G/G) and G-non carriers (GG/GG) genotypes is shown. Whiskers indicate variability outside the upper and lower quartile. Outliers are reported as individual dots. The average value is indicated by an x. The distributional difference was not significant on Student's t-test ($p > 0.05$).

For estimating the and NMS risk (genetic susceptibility) among G-carrier patients who developed the syndrome following antipsychotic intake, logistic regression models were adopted. The results showed that patients with G/G or G/GG genotype (G-carriers) had a 7.07 times greater risk of developing NMS than those with GG /GG genotype (G-non-carriers) controlling for age and sex (OR = 7.074, 95% CI = 1.414–35.379; $p = 0.017$). Thus, variant rs1799732 may be a putative biomarker associated with a greater risk of developing NMS upon antipsychotic therapy, although the estimate is inaccurate (wide odds ratio confidence interval) due to the small sample size (12 cases). However, NMS is a rare syndrome which, according to recent incidence estimates, occurs in 0.02-0.04% of patients treated with antipsychotics (Stübner *et al.*, 2004; Nielsen *et al.*, 2012; Schneider *et al.*, 2020). Over the duration of this three-year study, from 2017 to 2020, we

enrolled 12 of NMS cases, of which 5 had G/GG or G/G genotype, whereas among the 69 controls, only 9 carry G/GG or G/G genotype.

Notably, aripiprazole, a second generation antipsychotic, was the trigger in 4 out of 5 NMS patients with the G/GG genotype (G-carriers), whereas the haloperidol, a first generation antipsychotic, was the trigger in only 1 G-carrier patient. Conversely, haloperidol was the trigger in 4 out of 7 NMS patients with GG/GG genotype (non-G-carriers), of which 1 patient was being treated with aripiprazole and another one with clozapine. Overall, these two antipsychotics are the triggers in 11 out of 12 NMS (91.6%) patients, however these results indicate an opposite association between trigger and genotypes, with aripiprazole mainly associated to G/GG genotype (80%), and haloperidol to GG/GG genotype corresponding to about 57%. Moreover, of the two subjects who experienced recurrent episodes of NMS upon treatment with different APs administered as monotherapy with both FGA and SGA, 1 showed G-carrier genotype. The G-carrier genotype was observed in only 1 out of 4 patients that had elevated environmental temperature as a cofactor risk. Variation of the treatment plan, another cofactor of risk, was observed in 2 G-carriers out of 5 NMS patients. Polypharmacy also played a role as a cofactor of risk only in 3 G-carriers out of 8 NMS patients treated with cocktails of drugs (**Figure 33**).

In contrast, G/G or G/GG genotypes (G-carriers) does not seem to be associated with NMS-like symptoms due to exposure to substances of abuse, indeed among 13 patients only 2 are G-carriers, whereas the other 11 are G-non carriers (OR = 0.351, 95% CI = 0.021–5.886; $p = 0.467$).

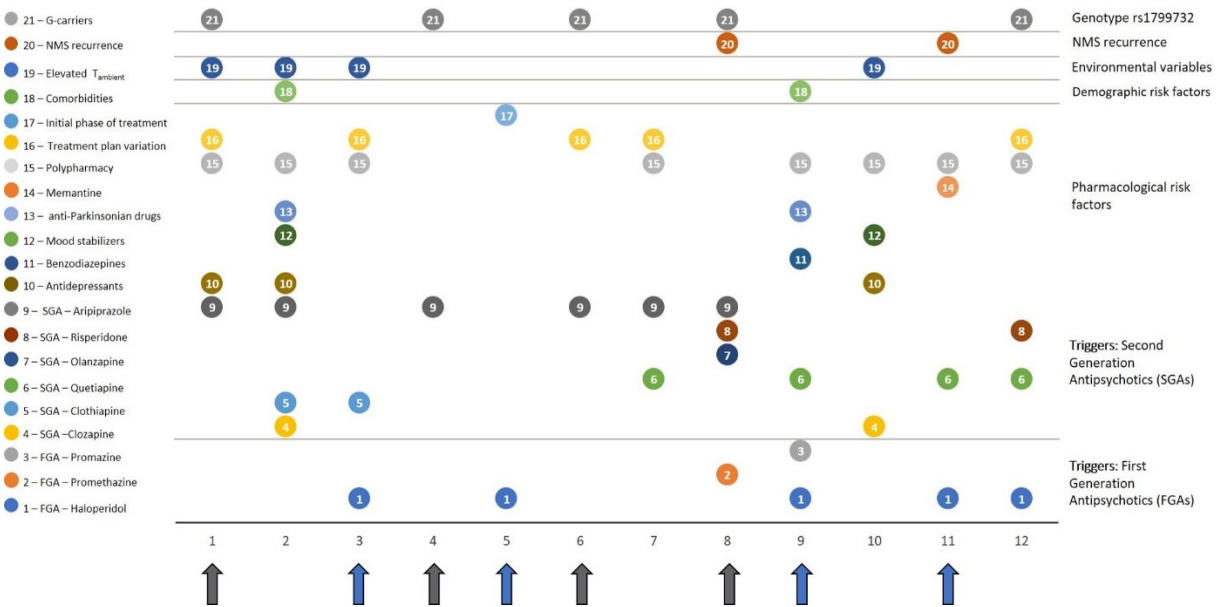

Figure 33. Triggers, risk factors, and rs1799732 genotype occurrence among NMS patients. Diagram summarizing triggers, risk factors and rs1799732 variant genotypes for the cohort of NMS patients is shown. Each trigger and risk factor has been assigned a numbered and coloured dot as specified on the left. On the abscissa axis NMS patients from 1 to 12 are reported. Arrows indicate G-carrier (G/G + G/GG) patients who were treated with aripiprazole (in dark grey) and G-non carrier (GG/GG) patients who were treated with haloperidol (in blue).

Cocaine and MDMA played a major role as substances of abuse triggering NMS-like symptoms. Indeed, cocaine was involved in 7 cases, whereas MDMA or its derivatives were involved in 5 cases. One patient developed NMS-like symptoms upon intake of both MDMA and cocaine. No association can be drawn between G/G or G/GG genotypes (G-carriers) and causative agents of NMS-like symptoms. Indeed, only 2 out of 13 NMS-like patients were G-carriers, one of which developed the syndrome upon MDMA intake, whereas the other one developed the syndrome upon assumption of atropine and cathinones (**Figure 34**).

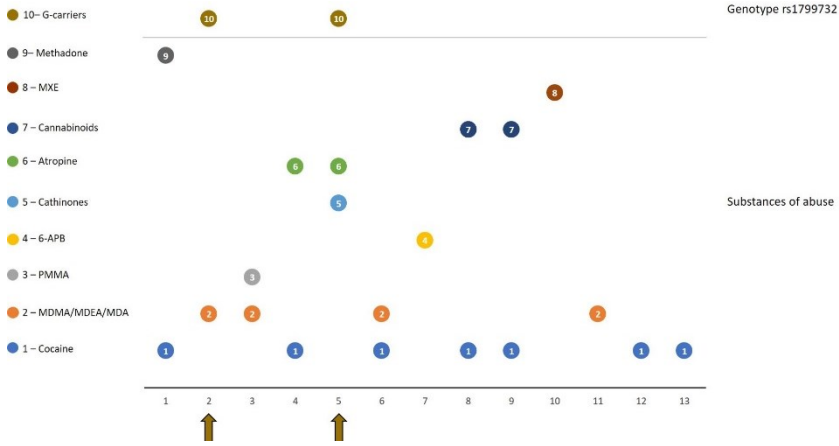

Figure 34. Triggers and rs1799732 genotypes among NMS-like patients. Diagram summarizing triggers and rs1799732 variant genotypes for the cohort of NMS-like patients is shown. Each trigger has been assigned a numbered and coloured dot as specified on the left. On the abscissa axis NMS-like patients from 1 to 13 are reported. Gold arrows indicate the two subjects with G-carrier genotype.

In conclusion, the G-allele variant of the rs1799732 SNP is significantly associated to a 7-fold increased susceptibility to NMS triggered by antipsychotics treatment, although the current size of the cohort needs be extended. In contrast, no association was found between the G-allele and NMS-like symptoms due to abuse of substance, suggesting a different triggering mechanism for the two conditions. Therefore, the variant rs1799732 could be a promising pharmacogenetic biomarker to be investigated in patients undergoing to antipsychotic therapy.

4.2.3.1 Bioinformatics analysis of TF binding sites affected by rs1799732 variant in the *DRD2* promoter region

The genotype analysis of the rs1799732 SNP showed that the reference allele G (G-carrier) is significantly associated with a 7-fold risk of developing NMS upon antipsychotics intake. Reduced levels of dopamine metabolite in the cerebrospinal fluid 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 in the brain may be a critical step for the onset of NMS (Nisijima and Ishiguro, 1995; Jauss *et al.*, 1996; Mann *et al.*, 2000). Reduced *DRD2* transcription levels have been reported by *in vitro* assay for the G-allele of this variant (Arinami *et al.*, 1997). However, this finding has not been confirmed by *in vivo* studies (Jönsson *et al.*, 1999; Pohjalainen *et al.*, 1999). With the purpose of further investigate if this variant exerts a regulatory function in human brain, a bioinformatics search for putative transcription factors binding sites in the promoter region of *DRD2* gene, in presence or in absence of the allele G, was performed by the free available software PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and AliBaba2.1(http://gene-regulation.com/pub/programs/alibaba2/index.html) (Messeguer *et al.*, 2002; Farré *et al.*, 2003; Matys *et al.*, 2003).

Results retrieved from the search performed by PROMO bioinformatic tools showed that the sequence flanking rs1799732 in presence of allele GG contains putative binding sites for transcription factors, such as AP2-alpha, TFII-I, Stat-4, and C-ets-1, whereas the G-allele lacks TFII-I, Stat-4, and C-ets-1binding sites, but generates a new Foxp3 binding site (PROMO, using version 8.3 of TRANSFAC). In particular, *STAT4* gene encodes the protein Signal transducer and activator of transcription 4 (Stat-4) that belongs to STAT family of transcription factors and works either as a signal transduction factor and as transcriptional activator (https://www.uniprot.org/uniprot/Q14765, https://www.genecards.org/cgi-bin/carddisp.pl?gene=STAT4). So far, it has been studied mainly in relation to immune system and autoimmune diseases. However, both protein and RNA encoded by *STAT4* are expressed in the brain (Human Protein Atlas,

https://www.proteinatlas.org/ENSG00000138378-STAT4/tissue). Moreover, according to the Consensus Human brain data set that reports normalized expression levels for the 10 brain regions obtained by combining the data from the two transcriptomics datasets GTEx and FANTOM5, *STAT4* RNA is expressed in both hypothalamus and basal ganglia, brain structures strictly involved in dopaminergic signalling pathways and especially in motor control and thermoregulation (**Figure 35**).

In addition, also a search by AliBaba2.1 software was performed. Results showed that the sequence flanking rs1799732 in presence of allele GG contains a putative binding site for the Transcriptional repressor protein YY1 (YY-1), encoded by *YY1* gene, that lacks when G-allele is present (AliBaba2.1). Transcriptional repressor protein YY1 is ubiquitously expressed in human tissue (**Figure 36**) and it is known to play a major role in the development of CNS, acting both as a transcriptional activator o repressor according to the cellular context (Verheul *et al.*, 2020).

Thus, lack of binding sites for the transcriptional factors Stat-4 or YY-1 in presence of allele G, might induce a reduction in D_2 receptor expression on the cellular membrane. Patients with a lower density of D_2 might be a higher risk of receptor over-blocking, thus needing a lower dose of antipsychotic medications (Oishi *et al.*, 2018).

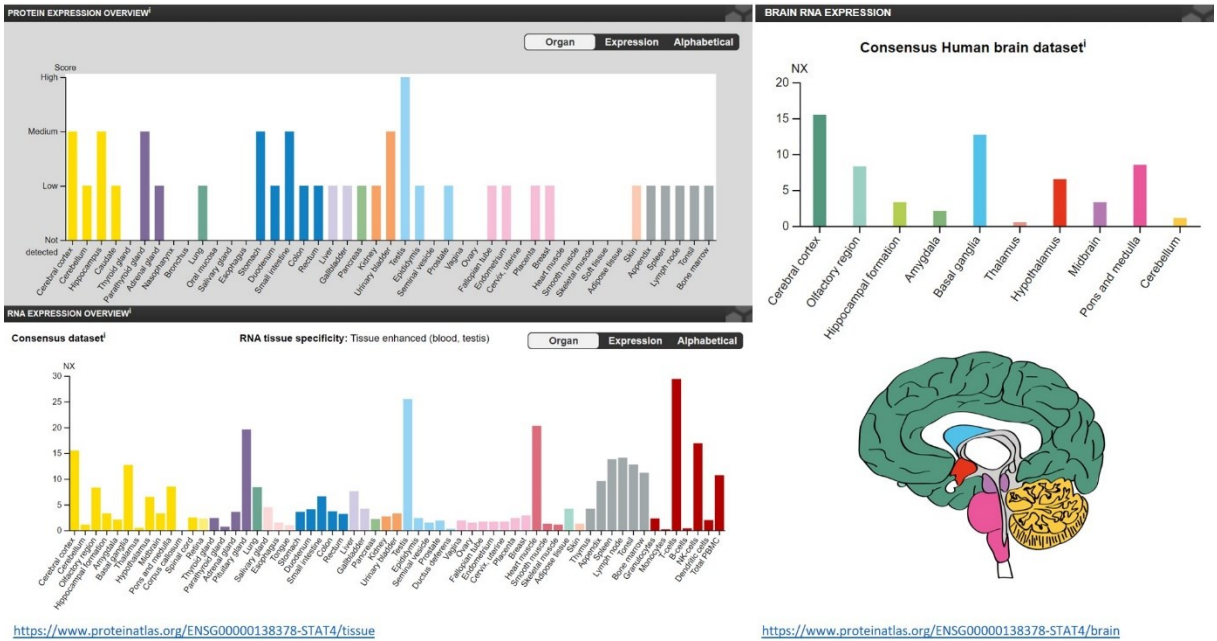

Figure 35. STAT4 RNA and protein expression. Protein and RNA expression for STAT4 is shown for all tissues on the left. The same color is used to indicate tissues with functional features in common. Bars show expression levels. STAT4 RNA expression in the main human brain regions in shown on the right. Color-coding is used to indicate the different brain regions. Bars show expression levels. (https://www.proteinatlas.org/).

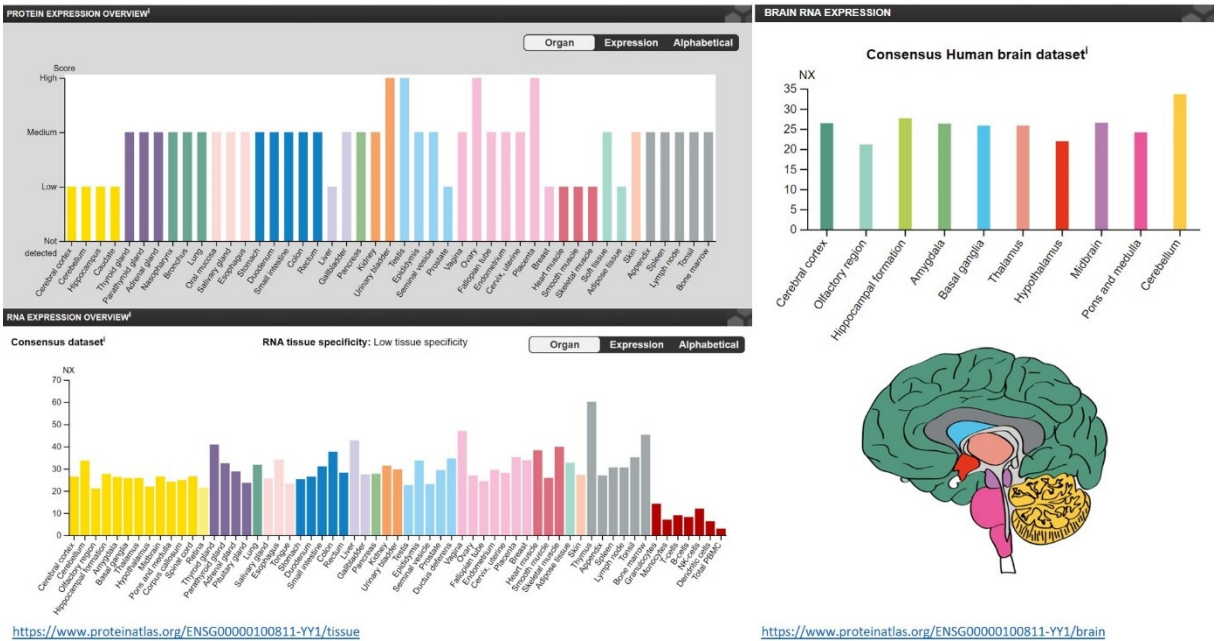

Figure 36. *YY1* RNA and protein expression. Protein and RNA expression for *YY1* is shown for all tissues on the left. The same color is used to indicate tissues with functional features in common. Bars show expression levels. YY1 RNA expression in the main human brain regions in shown on the right. Color-coding is used to indicate the different brain regions. Bars show expression levels (https://www.proteinatlas.org/).

5 DISCUSSION

The present study was carried out on Southern European patients to unravel a putative genetic predisposition to develop adverse reactions triggered by antipsychotics or abuse of illicit substances leading to hyperthermia and other clinical signs.

All the 25 patients included in the study presented the signs and symptoms commonly associated to NMS: hyperthermia resistant to pharmacological treatment with antipyretics and NSAIDs, muscle rigidity, rhabdomyolysis, mental status alteration, signs of SNS lability, hypermetabolism and negative workup for other aetiologies. However, causative agents were antipsychotics in 12 patients who developed NMS, and illicit substances of abuse in 13 patients who developed NMS-like symptoms.

It is generally accepted that central dopaminergic blockade, especially of dopamine receptor D_2, may be a critical step for the onset of NMS (Strawn, Keck and Caroff, 2007; Berman, 2011; Velamoor, 2017). Dysregulations of the dopaminergic signalling mediated by D_2 receptor have been also associated with Parkinson disease, schizophrenia and bipolar disorder (Mi *et al.*, 2011).

A common pathogenetic mechanism involving D_2 receptor has been also proposed to explain NMS-like symptoms that, in some instances, are described in association to the intake of serotonergic agents being both therapeutic drugs or illicit substances, such as cocaine or MDMA and related compounds (Daras *et al.*, 1995; Demirkiran, Jankovic and Dean, 1996; Wetli, Mash and Karch, 1996; Russell *et al.*, 2012; Mazhar *et al.*, 2016; Werneke *et al.*, 2016). It has been suggested that chronic cocaine abuse may affect dopaminergic neurotransmission either by dopamine depletion due to reuptake inhibition or by a decrease in the number of postsynaptic dopamine receptors, to compensate for the overstimulation of the dopaminergic system (Dackis and Gold, 1985; Volkow *et al.*, 1990). Moreover, it has been observed that the increased stimulation of serotonin receptors inhibits dopamine release (Steele, Keltner and McGuiness, 2011).

DRD2 gene encodes for the D_2 dopamine receptor, a G protein-coupled receptor located both pre- and post-synapsis on dopaminergic neurons of CNS and it is involved in physiological functions related to thermoregulation, muscle tone and locomotion, behavior, cognitive functions, and endocrine activity (Missale *et al.*, 1998; Balthazar *et al.*, 2010; Beaulieu and Gainetdinov, 2011). Thus, studies aimed at investigating genetic variants of *DRD2* gene associated to hyperthermic adverse reactions have been addressed to examine the genetic variants, namely rs1800497 and rs1799732, for putative association to NMS susceptibility in a Japanese population, although with conflicting results (reviewed in Kishida *et al.*, 2003). A third variant, the rs1799978, was also associated with antipsychotic treatment response (Arinami *et al.*, 1997; Xing *et al.*, 2007; Zhang, Lencz and Malhotra, 2010).

In the present study, the genetic variants rs1800497, that is mapped at about 10kb at the 5' side of the *DRD2* locus, although statistically irrelevant, showed a slight increase of the MAF alleles among the patients in comparison to control individuals. Historically, this variant was mapped within *DRD2* gene and it was suggested that it may affect *DRD2* gene regulation (Grandy *et al.*, 1991; Neville, Johnstone and Walton, 2004; Gluskin and Mickey, 2016). According to the GRCh38.p13 release of the human reference genome, the rs1800497 is, however, a missense variant that maps at position 11:113400106 into exon 8 of the adjacent *ANKK1* gene that encodes for ankyrin repeat and protein kinase domain-containing protein 1. The variant rs1800497 has been studied above all in relation to vulnerability to addictive behaviour and to antipsychotic drugs treatment response (Mi *et al.*, 2011; Naumovska *et al.*, 2015). Only a few pharmacogenetic studies investigated the role of *DRD2* Taq1A polymorphisms in relation to vulnerability to NMS, with conflicting results (Suzuki *et al.*, 2001; Kishida *et al.*, 2003, 2004; Mihara *et al.*, 2003). Two Japanese studies performed on a cohort of 15 and 17 patients who developed NMS upon antipsychotic treatment, respectively, suggested a putative association between NMS susceptibility and rs1800497. Nevertheless, the second study, was an extension of the previous one, since 13 patients had already been counted in the first study (Suzuki *et al.*, 2001; Mihara *et al.*, 2003). Two additional studies from another research group

performed on larger samples of patients (49 and 32 Japanese patients with NMS, respectively) did not observe any association of this variant with the NMS onset (Kishida *et al.*, 2003, 2004). On the contrary, this SNP has been associated with decreased glucose metabolism in putamen and several other cortical areas with dopaminergic innervation and lower dopamine receptor density in the striatum (Noble, Blum and Khalsa, 1991; Thompson *et al.*, 1997; Pohjalainen *et al.*, 1998; Jönsson *et al.*, 1999; Hirvonen, Laakso, *et al.*, 2009; Hirvonen, Lumme, *et al.*, 2009a; Savitz *et al.*, 2013; Gluskin and Mickey, 2016).

In accordance with Kishida *et al*., no association was observed in the current study by investigating rs1800497 on 12 NMS patients and 13 NMS-like patients belonging to Southern European Non-Finnish population.

The second variant, the rs1799978, investigated in the present study was historically named A-241G, since it resulted from a A to G substitution (reverse strand) on the promoter sequence of *DRD2* gene, and it was associated with antipsychotic treatment response (Xing *et al.*, 2007; Zhang, Lencz and Malhotra, 2010).

In our cohort, it showed a slight decrease of the MAF in the patients compared to control individuals, however this variation was not yet statistically significant, leading to exclude any association of the variant to NMS or NMS-like diseases.

The third SNP, rs1799732, was deeply investigated because of the low frequency (0.072) observed in our cohort of control individuals that agreed with the 0.076 minor allele frequency (MAF) reported for the Southern European population by gnomAD.

Among the overall patients of this study, this genetic marker showed about a 2-fold increase (0.140), however the statistically significant evidence was observed on NMS cohort alone. Indeed, among the 12 patients who developed NMS triggered by antipsychotic administration, 5 had the G/GG genotype (G-carrier), whereas the remaining 7 had the GG/GG genotype (G-non-carrier). This resulted in a strong increase of G-carrier frequency of about 3.2-

fold over control individuals. Contrary, only 2 out of 13 patients who developed NMS-like symptoms upon abuse of illicit drugs carried the reference allele G, resulting in no association.

Our results clearly show an association of this SNP with the NMS cohort alone. Indeed, the odds ratio estimated by logistic regression model resulted in a 7.07-fold times greater risk of developing NMS for patients with G/G or G/GG genotype (G-carriers) over the GG/GG genotype (G-non carriers). Importantly, gender and age were not confounding elements or effect modifiers since no significant distributional difference was found for gender or age between G-carrier and G-non carrier individuals.

Moreover, the G-carrier genotype did not show any association with NMS-like symptoms due to exposure to drug of abuse.

NMS is a rare syndrome occurring in 0.02-0.04% of patients treated with antipsychotics (Stübner *et al.*, 2004; Nielsen *et al.*, 2012; Schneider *et al.*, 2020). Over the duration of this three-year study, from 2017 to 2020, we enrolled 12 of NMS cases, of which 5 had G/GG or G/G genotype, whereas among the 69 controls, only 9 carry G/GG or G/G genotype. Thus, variant rs1799732 may be a putative biomarker associated with a greater risk of developing NMS upon antipsychotic therapy, although the estimate needs to be implemented by increasing the number of patients enrolled in the study.

Aripiprazole, a second generation antipsychotic (SGAs), was the most represented antipsychotic among NMS patients, followed by the FGA haloperidol. Notably, aripiprazole was the trigger in 4 out of 5 NMS patients with the G/GG genotype (G-carriers), whereas haloperidol was the trigger in only 1 G-carrier patient. Conversely, haloperidol was the trigger in 4 out of 7 NMS patients with GG/GG genotype (non-G-carriers), of which 1 patient was being treated with aripiprazole and another one with clozapine. Overall, these two antipsychotics are the triggers in 11 out of 12 NMS (91.6%) patients, however these results indicated that aripiprazole was mainly associated to G/GG genotype (80%), while haloperidol was the main trigger associated with GG/GG genotype, corresponding to about 57%.

Haloperidol is a first-generation antipsychotic (FGA) with high affinity for the D_2 receptor, known to be associated with NMS since early clinical study in 1960 (Delay *et al.*, 1960; Caroff and Mann, 1988). PET studies suggested that 60–80% of D_2 occupancy is needed for FGAs to produce a therapeutic response, while the 75–80% of D_2 receptor occupancy is related to EPS onset (Kapur and Mamo, 2003). Thus, it is very difficult to avoid the risk of overlapping the therapeutic dose of FGAs required for the D_2 receptor occupancy and that causing adverse reactions (Divac *et al.*, 2014).

In contrast, aripiprazole is a second-generation antipsychotic (SGA) and they are generally considered having potent antagonist activity at the 5-HT_{2A} receptor while showing lower affinity for D_2 receptor, accounting for fewer extrapyramidal symptoms than FGAs (Kapur and Mamo, 2003; Nasrallah, 2008; Brunton, Hilal-Dandan and Knollmann, 2017).

Given their receptor binding profile, SGAs were initially thought not to cause NMS (Pileggi and Cook, 2016). However, aripiprazole is mainly a partial agonist with a high affinity for D_2 pre- and post- synaptic receptors and it requires an elevated D_2 occupancy level (80%–95%) to obtain a therapeuthic effect (Yokoi *et al.*, 2002; Roth, Sheffer and Kroeze, 2004; Brunton, Hilal-Dandan and Knollmann, 2017; Tuplin and Holahan, 2017; Gonsai *et al.*, 2018). Moreover, it should be considered that SGAs prescriptions are raising and virtually all antipsychotics have been associated with NMS (Caroff and Mann, 1993; Morrens *et al.*, 2015; Schneider *et al.*, 2020). Indeed, many cases of NMS following exposure to second generation antipsychotics have been reported (Trollor *et al.*, 2012; Belvederi Murri *et al.*, 2015; Tse *et al.*, 2015; Singhai, Kuppili and Nebhinani, 2019).

Common risk factors, already described in literature as suspected to increase the probability of developing NMS, were observed among our cohort of NMS patients (Caroff and Mann, 1988, 1993; Viejo *et al.*, 2003; Tse *et al.*, 2015). The G-carrier genotype was observed in only 1 out of 4 patients that had elevated environmental temperature as a cofactor risk; among the 5 NMS patients who had variation of the treatment plan, only 2 were G-carriers; whereas, out of 8 NMS patients who experienced polypharmacy only 3 were

G-carriers. Notably, 2 patients experienced recurrent episodes of NMS upon treatment with different APs administered as monotherapy, being both FGA or SGA, as showing a sort of intrinsic vulnerability to antipsychotic treatment and 1 showed G-carrier genotype. In summary, no significant association was observed among risk factors and this polymorphism.

Among the patients who developed NMS-like symptoms following exposure to substances of abuse, cocaine played a major role, followed by MDMA and related compounds. However, no association of the rs1799732 SNP was found with NMS-like patients whose adverse reactions were triggered by abuse of illicit substances. Thus, the rs1799732 SNP appears to be in association to antipsychotics and not to illicit drugs suggesting that different biochemical mechanisms may be involved in the triggering the adverse reactions leading to common clinical signs such those observed for NMS or NMS-like symptoms.

The variant rs1799732 has been mapped into the *DRD2* promoter region, therefore a preliminary analysis of TF consensus binding sites was carried out by PROMO and AliBaba2.1 bioinformatic tools (Messeguer *et al.*, 2002; Farré *et al.*, 2003; Matys *et al.*, 2003). PROMO identified on GG allele of this SNP putative binding sites for transcription factors, such as TFII-I, Stat-4, and c-Ets-1, that were absent in the G-allele and replaced by Foxp3 binding site. Interestingly, Stat-4 has been described as a signal transduction factor and transcriptional activator that is expressed in the brain, especially in basal ganglia and hypothalamus, regions that play a critical role in the control of basal temperature of the body and motor functions. Moreover, AliBaba2.1 found the putative binding site for the transcription factor YY-1 in the GG allele of this SNP, that lacked in the G-allele. YY-1 transcription factor is ubiquitously expressed in human cells and it is known to play a major role in the development of CNS acting both as a transcriptional activator o repressor according to the cellular context (Verheul *et al.*, 2020). Thus, an intriguing hypothesis is that lack of binding sites for Stat-4 or YY-1 transcription factors in presence of allele G might induce a reduction in D_2 receptor expression on the cellular membrane. Patients with a lower density of D_2 might have a higher risk of receptor over-blocking, thus needing a lower dose of

antipsychotic medications (Oishi *et al.*, 2018). Consistently, a reduced density of D_2 receptor has been reported in association to this variant by *in vitro* luciferase reporter assay (Arinami *et al.*, 1997). This observation was not confirmed by *in vivo* PET studies using [^{11}C]raclopride on healthy volunteers in both striatal and extra-striatal brain regions (EG Jönsson *et al.*, 1999; Pohjalainen *et al.*, 1999; Hirvonen, Laakso, *et al.*, 2009b). However, reduced levels of the dopamine metabolite HVA in the cerebrospinal fluid of patients with NMS and a marked reduction – by 50% to 70% - of levels of HVA in the striatum of a patient suspected of having died for NMS, lack of D_2 receptor binding activity and functional alterations of the dopaminergic system during NMS have been described (Kish *et al.*, 1990; De Reuck *et al.*, 1991; Nisijima and Ishiguro, 1995; Jauss *et al.*, 1996). Further functional studies are needed to understand the molecular events that lead to NMS and the putative role of rs1799732 in NMS susceptibility.

In conclusion, data suggested that the rs1799732 may be a promising pharmacogenetic biomarker of NMS susceptibility associated with a greater risk of developing NMS in patients being treated with antipsychotics. Different biochemical mechanisms appear to trigger the adverse reaction of NMS and NMS-like symptoms by antipsychotics vs illicit substances. Lastly, intriguing is the susceptibility to NMS by the aripiprazole treatment in G-allele carrier patients. This study may become useful in the clinical practice to personalize the antipsychotic treatment with the purpose of maximizing efficacy and reducing adverse reactions.

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