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METABOLIC ADVERSE EFFECTS INDUCED BY SECOND GENERATION ANTIPSYCHOTIC DRUGS IN PEDIATRIC PATIENTS: PHARMACOGENETIC PROFILING AND MECHANISTIC ANALYSIS OF GENETIC VARIANTS EFFECT IN *VITRO* MODELS

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ABSTRACT

Second-generation antipsychotics (SGAs) are increasingly used in pediatric patients both in and off-label. The rise in SGA prescription rates may be due to the perception of improved safety compared with first-generation antipsychotics. However, SGA use is often associated with rapid weight gain and metabolic syndrome as adverse drug reactions (ADRs). Interestingly, these side effects are not observed in all SGA-treated children, suggesting that some underlying genetic factors may predispose an individual to develop these adverse conditions.

Recent studies have identified single nucleotide polymorphisms (SNPs) in genes encoding for enzymes involved in drug metabolism (CYP450) and SGA receptors in the central nervous system (CNS), which partly explain the inter-patient variability in ADR development.

With this project, we intend to define the role of SNPs in genes related to the pharmacodynamics of SGA, expressed in peripheral tissues, to evaluate their involvement in the mechanisms responsible for weight change and metabolic ADRs.

To this end in a cohort of 209 paediatric patients affected by disruptive behavioural disturbance who are prescribed SGAs (risperidone and aripiprazole), we have assessed the association of pharmacodynamics genetic variants with the occurrence of changes in body mass index (BMI)- Z score. This allows us to evaluate if the presence of specific variant alleles increases or decreases the risk of weight changes and metabolic adverse events.

Tested SNPs will be selected from a comprehensive meta-analysis by Zhang e al (2016) of associated gene variants with antipsychotic-related weight gain. Between the 15 SNPs studied, the variants statistically significant associated with BMI changes are rs6318, rs3813929, rs5181147 in 5-Hydroxytryptamine Receptor 2C (HTR2C), rs1799732, rs6275, rs7131056 in D2 dopamine receptor (DRD2) and rs489693 in melanocortin-4-receptor (MCR4). We consider that the identification of these SNPs might provide useful input for personalized and individualized early intervention.

Another goal of this project is to examine the direct effect of SGAs and validate the role of selected SNP at the peripheral level by performing in vitro experiments.

Dysregulation in adipose tissue homeostasis has been suggested as a plausible mechanism by which antipsychotics induced metabolic alteration. To investigate the impact of SGAs on dysfunctional adipose tissue we decided to use the SW872 liposarcoma cell model.

First, we performed a detailed characterization of the model establishing the cell culture condition for the adipogenic differentiation process.

Then, to clarify the mechanisms by which SGAs induce weight gain we studied the risperidone effect on the adipogenesis of SW872. We observed that risperidone directly promoted adipocyte differentiation and lipid accumulation.

To better understand the phenotype of risperidone-induced adipocytes, next we assess its impact on some mitochondria parameters. We observed that risperidone's effect on mitochondrial phenotype can more faithfully recapitulate the white adipocyte profile observed in human adipose tissue.

In line with this, our data also showed that Hoxc4, a key marker of white adipocytes, is highly expressed in risperidone adipocytes.

From these results, we hypothesize that the induction of white adipocytes may be a possible mechanism by which risperidone induces weight gain as a side effect.

Finally, to validate in the adipocyte cell line the impact of SNPs on differentiation, we have set up an in vitro model system for the expression of the genetic variants, by choosing the site-directed mutagenesis approach to modified the DNA sequence of the genes of interest . We selected two SNPs: rs6318 for the 5-HT2C gene and rs6275 for the DRD2 gene, identified in our population study as related to BMI changes after SGAs administration and already evaluated in correlation with weight gain, metabolic ADRs, and antipsychotic treatment with a focus on SNC expression . Using our adipocyte differentiation protocol, we showed that the modified cells could be differentiated into adipocytes therefore providing a relevant model for studying the SNP role in SGAs-induced adipogenesis.

SINTESI

Gli antipsicotici di seconda generazione (SGA) sono sempre più utilizzati in età pediatrica, sia in regime di inibizione che off-label. L'aumento delle prescrizioni di SGA può essere dovuto alla percezione di una maggiore sicurezza rispetto agli antipsicotici di prima generazione. Tuttavia, l'uso di SGA è spesso associato a un rapido aumento di peso e alla sindrome metabolica come reazioni avverse al farmaco (ADR). È interessante notare che questi effetti collaterali non si osservano in tutti i bambini trattati con SGA, suggerendo che alcuni fattori genetici sottostanti possono predisporre un individuo a sviluppare queste condizioni avverse.

Studi recenti hanno identificato polimorfismi a singolo nucleotide (SNP) nei geni che codificano per enzimi coinvolti nel metabolismo dei farmaci (CYP450) e nei recettori dei SGA nel sistema nervoso centrale (SNC), che spiegano in parte la variabilità tra i pazienti nello sviluppo di ADR.

Con questo progetto, intendiamo definire il ruolo degli SNP nei geni correlati alla farmacodinamica degli SGA, espressi nei tessuti periferici, per valutare il loro coinvolgimento nei meccanismi responsabili del cambiamento di peso e delle ADR metaboliche.

A tal fine, in una coorte di 209 pazienti pediatrici affetti da disturbi comportamentali a cui sono stati prescritti SGA (risperidone e aripiprazolo), abbiamo valutato l'associazione delle varianti genetiche farmacodinamiche con il verificarsi di variazioni dell'indice di massa corporea (BMI)-Z score. Questo ci permette di valutare se la presenza di varianti alleliche specifiche aumenta o diminuisce il rischio di variazioni di peso e di eventi avversi metabolici.

Gli SNP testati sono stati selezionati da una meta-analisi completa di Zhang e al. (2016) sulle varianti geniche associate all'aumento di peso correlato agli antipsicotici. Tra i 15 SNP studiati, le varianti associate in modo statisticamente significativo alle variazioni di BMI sono rs6318, rs3813929, rs5181147 nel recettore 2C della 5-idrossitriptamina (HTR2C), rs1799732, rs6275, rs7131056 nel recettore della dopamina D2 (DRD2) e rs489693 nel recettore della melanocortina-4 (MCR4). Riteniamo che l'identificazione di questi SNP possa fornire utili indicazioni per un intervento precoce personalizzato e individualizzato.

Un altro obiettivo di questo progetto è esaminare l'effetto diretto degli SGA e convalidare il ruolo degli SNP selezionati a livello periferico eseguendo esperimenti in vitro.

La disregolazione dell'omeostasi del tessuto adiposo è stata suggerita come meccanismo plausibile per l'alterazione metabolica indotta dagli antipsicotici. Per studiare l'impatto degli SGA sulle

disfunzioni del tessuto adiposo, abbiamo deciso di utilizzare come modello cellulare le SW872 una linea cellulare di liposarcoma.

In primo luogo, abbiamo eseguito una caratterizzazione dettagliata del modello, stabilendo le condizioni di coltura delle cellule per il processo di differenziazione adipogenico.

Poi, per chiarire i meccanismi con cui gli SGA inducono l'aumento di peso, abbiamo studiato l'effetto del risperidone sull'adipogenesi delle SW872. Abbiamo osservato che il risperidone promuoveva direttamente la differenziazione degli adipociti e l'accumulo di lipidi.

Per comprendere meglio il fenotipo degli adipociti indotti dal risperidone, abbiamo poi valutato il suo impatto su alcuni parametri mitocondriali. Abbiamo osservato che l'effetto del risperidone sul fenotipo mitocondriale può ricapitolare più fedelmente il profilo degli adipociti bianchi osservato nel tessuto adiposo umano.

In linea con ciò, i nostri dati hanno anche mostrato che Hoxc4, un marcatore chiave degli adipociti bianchi, è altamente espresso negli adipociti con risperidone.

Da questi risultati, ipotizziamo che l'induzione degli adipociti bianchi possa essere un possibile meccanismo attraverso il quale il risperidone induce l'aumento di peso come effetto collaterale.

Infine, per convalidare nella linea cellulare adipocitaria l'impatto degli SNP sul differenziamento, abbiamo allestito un sistema modello in vitro per l'espressione delle varianti genetiche, scegliendo l'approccio della mutagenesi sito-specifica per modificare la sequenza del DNA dei geni di interesse. Abbiamo selezionato due SNP: rs6318 per il gene 5-HT2C e rs6275 per il gene DRD2, identificati nel nostro studio di popolazione come correlati alle variazioni di BMI dopo la somministrazione di SGA e già valutati in correlazione con l'aumento di peso, le ADR metaboliche e il trattamento antipsicotico, con particolare attenzione a livello del SNC. Utilizzando il nostro protocollo di differenziazione degli adipociti, abbiamo dimostrato che le cellule modificate potevano essere differenziate in adipociti, fornendo quindi un modello rilevante per studiare il ruolo degli SNP nell'adipogenesi indotta dagli SGA.

INTRODUCTION

Chapter 1
SECOND GENERATION ANTIPSYCHOTICS IN CHILDREN AND ADOLESCENT: AN OVERVIEW

1.1 Uses and applications

The new class of antipsychotic medications called atypical antipsychotics or second-generation antipsychotics (SGAs), work in a way that is significantly different from that of the previous class of antipsychotics (e.g., haloperidol, Thorazine), known as first-generation antipsychotics (FGAs). SGAs offer superior safety and similar efficacy compared with conventional agents in adults with psychotic disorders. For this reason, atypical antipsychotics have been increasingly used in children and adolescents. These drugs are not interchangeable as each of them has a unique pharmacologic profile and may differ considerably in terms of adverse effects. Five agents, *i.e.* risperidone, aripiprazole, olanzapine, paliperidone, and quetiapine currently have been approved by the Food and Drug Administration (FDA) for use in children and adolescents for the treatment of schizophrenia, bipolar disorder (manic or mixed), and irritability with autistic disorders. Clinicians may first suggest a different type of drug, psychotherapy, or some other type of nonpharmacologic treatment before suggesting an antipsychotic in a young child (Newcomer, 2005) (Patel et al., 2005). At the same time, psychiatrists and family doctors regularly prescribe SGAs as "off-label" medications for behavioural control purposes. The drugs are helpful in reducing aggressive behaviours and emotional outbursts, helping Attention-Deficit/Hyperactivity Disorder (ADHD) youths go to sleep (i.e., to counteract the effects of stimulant medication), or making them less "impulsive." Physicians also prescribe them as adjunctive medications for anxiety and depression. A 2016 study has shown that two-thirds of paediatric prescriptions of antipsychotics in the USA were for off-label purposes (Fig.1) (Sohn et al., 2016).

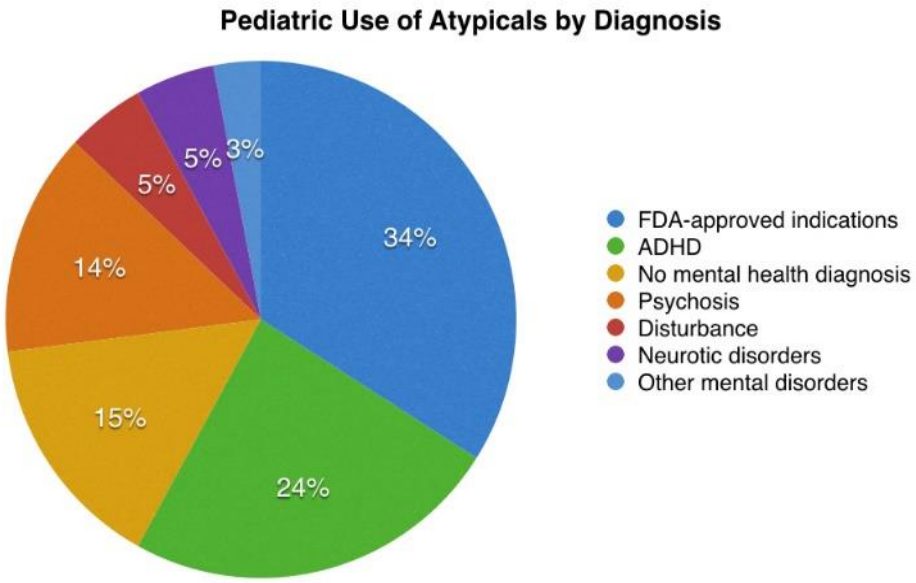

Figure 1. National trends in off-label use of atypical antipsychotics in children and adolescents in the United States (Sohn et al., 2016).

Therefore, SGAs are largely used for symptomatic treatment of pervasive developmental problems such as autism, Asperger syndrome and other autism spectrum disorders, Tourette syndrome, ADHD, and disruptive behaviour disorders such as oppositional defiant disorder and conduct disorders.
In terms of adverse effects, they have generally lower risks of extrapyramidal symptoms (EPS) and tardive dyskinesia compared to FGAs. However, these medications cause higher rates of weight gain and metabolic disorders (e.g., hypertension, hyperglycaemia, high cholesterol) that can be responsible for poor adherence, sub-optimal and discontinuation drug use, resulting in relapse and poor clinical outcome. Each individual drug varies for adverse effects and relevant metabolic side effects (Morrato et al., 2010).

1.2 The Spectrum of Atypia and the receptor binding profile of SGAs

Each individual drug in the class of SGAs vary in terms of efficacy motor adverse effects and relevant metabolic side effects. To explain this diversity among the SGAs, the concept of spectrum of atypia was recently introduced. SGAs were listed in three categories, starting with risperidone, the least atypical (Level I), and finishing with clozapine, the most atypical (Level III), with all others falling within these two extremes of the spectrum (Level II). Of note, risperidone and amisulpride can lose their atypicality at higher doses (Carli et al., 2021). With this classification, the concept of atypia refers to a category of APs demonstrating reduced motor problems, reduced hyperprolactinemia, and

reduced worsening of apathy and anhedonia along with a better improvement of negative and cognitive symptoms of schizophrenia when compared with FGAs (Figure.2) (Carli et al., 2021).

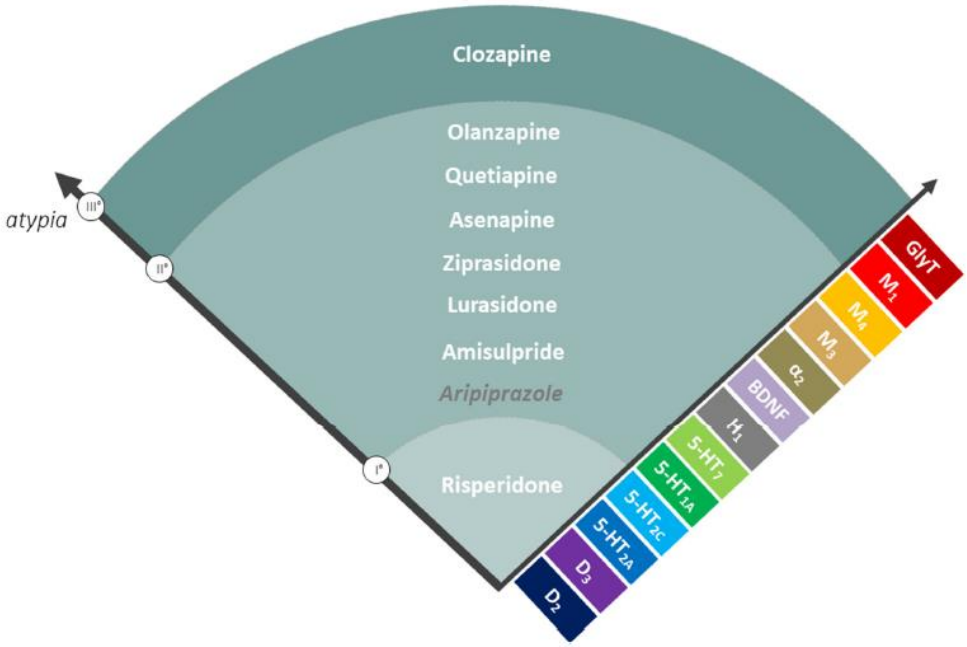

Figure 2. The concept of spectrum of atypia was recently introduced to classify atypical antipsychotics. The molecular targets are shown on the right (Carli et al., 2021).

Regarding the mechanism of action, SGAs are weak D2 dopamine receptor (DRD2) antagonists, and they act involving other receptor targets (*e.g.,* serotonin (5-HT) receptors (HTR)) (Scarselli et al., 2000)(Gillespie et al., 2017). A rapid dissociation constant (Koff) from the DRD2 along with the ratio of HTR2A/DRD2 and HTR2C/DRD2 affinity are two important factors that identify SGAs in terms of efficacy and side effects. Moreover, or all SGAs (including clozapine), many works have pointed out the importance of other G protein-coupled receptors (GPCRs), beyond DRD2 and HTR2A and HTR2C, such as other serotonin (HTR1A, HTR6 and HTR7) and dopamine receptors (DRD1, DRD3 and DRD4), as well as the histamine receptor H1R, muscarinic receptors (MR1, MR2, MR3, MR4 and MR5) and adrenergic receptors (ADRA1A and ADRA2A) (Fasciani et al., 2020)(Nasrallah, 2008). The receptor-binding profiles of six marketed SGA drugs (aripiprazole, clozapine, olanzapine, quetiapine, risperidone and ziprasidone) are presented in figure 3 (Nasrallah, 2008).

	Aripiprazole	*Clozapine*	*Olanzapine*	*Quetiapine*	*Risperidone*	*Ziprasidone*
5-HT$_{1A}$ [a]	$8 < pK_i < 9$	$6 < pK_i < 7$	$pK_i < 6$	$6 < pK_i < 7$	$6 < pK_i < 7$	$7 < pK_i < 8$
5-HT$_{1B}$	$6 < pK_i < 7$	$6 < pK_i < 7$	$6 < pK_i < 7$	$pK_i < 6$	$8 < pK_i < 9$	$8 < pK_i < 9$
5-HT$_{1D}$	$7 < pK_i < 8$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$	$6 < pK_i < 7$	$8 < pK_i < 9$
5-HT$_{1E}$	$pK_i < 6$	$6 < pK_i < 7$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$
5-HT$_{2A}$	$8 < pK_i < 9$	$7 < pK_i < 8$	$7 < pK_i < 8$	$6 < pK_i < 7$	$pK_i > 9$	$8 < pK_i < 9$
5-HT$_{2C}$ [a,b]	$6 < pK_i < 7$	$7 < pK_i < 8$	$8 < pK_i < 9$	$pK_i < 6$	$7 < pK_i < 8$	$7 < pK_i < 8$
5-HT$_3$	$6 < pK_i < 7$	$6 < pK_i < 7$	$6 < pK_i < 7$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$
5-HT$_5$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$	$6 < pK_i < 7$	$6 < pK_i < 7$
5-HT$_6$	$6 < pK_i < 7$	$7 < pK_i < 8$	$8 < pK_i < 9$	$pK_i < 6$	$pK_i < 6$	$7 < pK_i < 8$
5-HT$_7$	$7 < pK_i < 8$	$7 < pK_i < 8$	$6 < pK_i < 7$	$6 < pK_i < 7$	$8 < pK_i < 9$	$8 < pK_i < 9$
D$_1$	$6 < pK_i < 7$	$6 < pK_i < 7$	$7 < pK_i < 8$	$6 < pK_i < 7$	$6 < pK_i < 7$	$7 < pK_i < 8$
D$_2$ [a]	$pK_i > 9$	$6 < pK_i < 7$	$7 < pK_i < 8$	$6 < pK_i < 7$	$8 < pK_i < 9$	$8 < pK_i < 9$
D$_3$	$8 < pK_i < 9$	$6 < pK_i < 7$	$7 < pK_i < 8$	$6 < pK_i < 7$	$7 < pK_i < 8$	$7 < pK_i < 8$
D$_4$	$6 < pK_i < 7$	$7 < pK_i < 8$	$7 < pK_i < 8$	$pK_i < 6$	$7 < pK_i < 8$	$6 < pK_i < 7$
D$_5$	$pK_i < 6$	$6 < pK_i < 7$	$7 < pK_i < 8$	$pK_i < 6$	$6 < pK_i < 7$	$6 < pK_i < 7$
α_{1A}	$7 < pK_i < 8$	$8 < pK_i < 9$	$6 < pK_i < 7$	$7 < pK_i < 8$	$8 < pK_i < 9$	$7 < pK_i < 8$
α_{1B}	$7 < pK_i < 8$	$8 < pK_i < 9$	$6 < pK_i < 7$	$7 < pK_i < 8$	$8 < pK_i < 9$	$8 < pK_i < 9$
α_{2A}	$7 < pK_i < 8$	$6 < pK_i < 7$	$6 < pK_i < 7$	$pK_i < 6$	$6 < pK_i < 7$	$6 < pK_i < 7$
α_{2B}	$6 < pK_i < 7$	$7 < pK_i < 8$	$7 < pK_i < 8$	$6 < pK_i < 7$	$6 < pK_i < 7$	$7 < pK_i < 8$
α_{2C}	$7 < pK_i < 8$	$7 < pK_i < 8$	$7 < pK_i < 8$	$7 < pK_i < 8$	$8 < pK_i < 9$	$7 < pK_i < 8$
M$_1$	$pK_i < 6$	$7 < pK_i < 8$	$7 < pK_i < 8$	$6 < pK_i < 7$	$pK_i < 6$	$pK_i < 6$
M$_2$	$pK_i < 6$	$7 < pK_i < 8$	$7 < pK_i < 8$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$
M$_3$ [b]	$pK_i < 6$	$7 < pK_i < 8$	$7 < pK_i < 8$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$
M$_4$	$pK_i < 6$	$7 < pK_i < 8$	$6 < pK_i < 7$	$6 < pK_i < 7$	$pK_i < 6$	$pK_i < 6$
M$_5$	$pK_i < 6$	$7 < pK_i < 8$	$8 < pK_i < 9$	$pK_i < 6$	$pK_i < 6$	$pK_i < 6$
H$_1$ [a,b]	$7 < pK_i < 8$	$8 < pK_i < 9$	$8 < pK_i < 9$	$8 < pK_i < 9$	$7 < pK_i < 8$	$6 < pK_i < 7$

Abbreviations: α, α-adrenoreceptor; D, dopamine receptor; H, histamine receptor; 5-HT, serotonin receptor; M, muscarinic acetylcholine receptor.
Adapted from Roth *et al.* (2004).[11]
[a]Associated with weight gain.[51,52]
[b]Associated with increased risk of diabetes.[52,82]

Figure 3. Atypical antipsychotic and their affinities for receptor subtype (Nasrallah, 2008).

In addition to having distinct binding profiles, some SGAs have a unique behaviour in terms of receptor activity. For instance, aripiprazole acts as partial dopamine agonist, with a lower intrinsic activity at the receptors compared to full agonists, and therefore not able to induce a maximal response. Considering this, partial agonists may act either as agonists or antagonists, depending on the surrounding levels of the endogenous neurotransmitter (Greenaway & Elbe, 2009).

Moreover, recently new mechanisms associated with GPCR function have been discovered. Among them, the ability of β-arrestin to activate multiple mediators like ERK, proto-oncogene tyrosine-protein kinase SRC, nuclear factor-kB, and phosphoinositide 3-kinase the "biased agonism", and receptor dimerization have added further complexity and intrigue over the mechanism of action of SGAs (Aringhieri et al., 2017).

Finally, besides GPCRs, to explain the features of SGAs other targets have also been considered, such as ion channels (e.g., N-methyl-D-aspartate (NMDA)), transporters (e.g., Glycine transporters) and enzymes (e.g., glycogen synthase kinase 3(GSK3)).

1.3 Antipsychotic Exposure in Youth and Recommended Doses of Antipsychotics

The need for effective therapeutic interventions for children and adolescents with neuropsychiatric conditions has led to the increasing prescription of SGAs.

In order to maximize the response and minimize the side effect, pharmacological treatment should consist of three phases: an acute phase, which includes the initiation of medication treatment and subsequent dose adjustments; continuation phase, during which patients responding to the treatment consolidate their gains and remission or recovery occurs; and a discontinuation phase during which, if medically appropriate, the medication is successfully tapered with minimal risk for relapse/recurrence.

The general approach to initiating SGAs, recommended by the American Academy of Child and Adolescent Psychiatry (AACAP), is to 'start low and go slow', and use the lowest effective dose. Further, the maximum dose used in children should not exceed maximum doses used in adults (Figure 4) includes maximum recommended doses for children and adolescents based on FDA-approved product and Maximum (oral) Dosage from the Literature (Frydrych et al., 2019).

			for pediatric indications later occurred)
Olanzapine[48]	Zyprexa® (tabs, powder for solution for IM use) Zyprexa Zydis®(oral dist. tab)	• Bipolar mania/mixed and schizophrenia (13-17 years): 20 mg/day • *Also indicated for bipolar depression in children/adolescents ages 10-17 years (max 12 mg) when used with fluoxetine (Symbyax)*	• Age 4-5 years: 12.5 mg/day • Age 6-17 years: 20 mg/day
Paliperidone[49]	Invega® (ER tablet)	• Schizophrenia (12-17 years): 6 mg/day for weight <51 kg; 12 mg/day for weight ≥ 12 mg/day	• Schizophrenia (Age ≥ 12 years): 6 mg/day for weight <51 kg; 12 mg/day for weight ≥ 12 mg/day • Insufficient evidence in children
Quetiapine[50]	Seroquel®, Seroquel XR (brand only)	• Bipolar mania (10-17 years): 600 mg/day • Schizophrenia (13-17 years): 800 mg/day	• Age 5-9 years: 400 mg/day • Age 10-17 years: 800 mg/day
Risperidone[51]	Risperdal®, Risperdal M-tab® (oral dist. tab), Risperdal® (oral soln)	• Schizophrenia (13-17 years) or acute bipolar mania/mixed (10-17 years): 6 mg/day • Irritability associated with autism (5-17 years): 3 mg/day	• Age 4-11 years: 3 mg/day • Age ≥ 12 years: 6 mg/day
Ziprasidone[52]	Geodon®	• Not FDA-approved in children or adolescents	• Bipolar disorder (10-17 years): 80 mg/day for weight ≤ 45 kg; 160 mg/day for weight > 45 kg • Tourette's disorder: 40 mg/day

Figure 4. Overview of FDA-approved indications for Antipsychotics in Children and Maximum (oral) Dosage from the Literature (Frydrych et al., 2019).

At present, risperidone and aripiprazole are labelled by the FDA for use in children or adolescents for irritability associated with autistic disorder (5-16 years for risperidone and 6-17 years for

aripiprazole). In addition, risperidone, aripiprazole, olanzapine, and quetiapine are FDA-approved for use in the treatment of adolescents with schizophrenia (ages 13-17) and youths between the ages of 10 and 17 years with bipolar I disorder suffering from mixed or manic episodes. Despite these limited FDA indications, SGAs are still commonly prescribed for the treatment of numerous other conditions in paediatric patients (Crystal et al., 2016) (Kealey et al., 2014).

1.4 Safety Concerns

SGAs initially emerged as an alternative treatment to ameliorate the side-effect profile associated with their therapeutic predecessors, the FGAs. Some SGAs have a lower propensity to cause and/or exacerbate extrapyramidal symptoms (e.g., akathisia, dystonia, Parkinsonism, tardive dyskinesia). The risk for metabolic side effect is more common with SGAs and more prevalent in children comparing to adults. In part, this may be explained because adolescents exhibit pharmacokinetic differences (e.g., greater glomerular filtration rates, lower protein binding, less fat tissue) which functionally necessitates higher doses per kilogram of weight to achieve the same effect compared to adults for some medications (Ben Amor, 2012).

Youth exposed to antipsychotics, especially SGAs, are at higher risk for developing side effects including weight gain, impaired glucose metabolism with possible development of type 2 diabetes mellitus, dyslipidaemia, and elevated prolactin levels (Lambert et al., 2018).

1.5 SGAs and metabolic adverse reaction

SGAs, Weight gain and Metabolic Syndrome

Weight gain is a typical and worrying side effect of SGAs in children and adolescents. In addition, problems such as lipid and glucose disproportion and cardiovascular diseases are linked to age-inappropriate weight increase. Moreover, SGA treatment-emergent weight gain significantly contributes to noncompliance, treatment discontinuation/ switching and increased risk for relapse.

According to a recent systematic review and meta-analysis, youth who take SGAs acquire more weight than those who take a placebo or none although the effects of different antipsychotics vary; for instance, not all SGAs cause greater weight gain, with Olanzapine associated with the highest risk (Shah et al., 2019). In a network meta-analysis, the greatest increase in BMI was observed with clozapine and olanzapine (with a mean difference versus placebo/no antipsychotic of 2.38 and 4.14 kg, respectively). Of note, an increase in body mass index (BMI) has also been reported during short-term treatments (Dayabandara et al., 2017).

Several studies have examined the relationship between neurotransmitter receptor-binding profiles and weight gain. One hypothesis, albeit not all evidence is in support, is that antagonism of the

histamine 1 receptor (H1R) and HTR2C is a key event in SGA-associated weight gain. For example, SGAs with high affinities for the H1R had the highest correlation with weight gain (Spearman's rho: $r = -0.72$; $p < 0.01$), followed by the ADRA1A (Spearman's rho: $r = -0.54$; $p < 0.05$), the 5-HT2C receptor (Spearman's rho: $r = -0.49$; $p < 0.05$) and the HTR6 (Spearman's rho: $r = -0.52$, $p < 0.005$) (Pillinger et al., 2020). Conversely, SGAs such as ziprasidone, aripiprazole, and lurasidone, with a low affinity for H1R in vitro, have been associated with a low risk for weight gain (Pillinger et al., 2020).

Another concern relevant to iatrogenic weight gain in mood-disorder patients is fat distribution and increased waist circumference. High waist circumference is associated with an increased risk for hypertension, diabetes mellitus, dyslipidaemia, and metabolic syndrome (MetS) (Panagiotopoulos et al., 2012). MetS is a significant problem for patients receiving antipsychotic drugs, as it is responsible for reduced life expectancy and poor adherence to the therapy. It is defined as a cluster of signs and symptoms, such as insulin resistance, dyslipidaemia, and hypertension, that increases the risk of type 2 diabetes, heart disease, and stroke. Results of a 2018 systematic review of 126 studies report that antipsychotic paediatric populations showed compared to placebo, elevated triglyceride levels, weight gain, increased risk of type 2 diabetes, and unfavourable lipid changes (Ben Amor, 2012). Consequently, part of these patients developed dyslipidaemia (17.1%), insulin resistance (8.6%), and MetS (1.6%). Antipsychotics can affect metabolic function through direct effects on lipids and insulin sensitivity and indirect effects on these parameters as a result of increased weight gain and obesity (Grajales et al., 2019).

Several reviews and meta-analysis that systematically compared SGAs with FGAs in terms of efficacy and side effects revealed data supporting SGAs superiority for several aspects, but with an increased risk of developing MetS (De Hert et al., 2008). As for weight gain, in terms of risk, SGAs are a heterogeneous class of drugs. As expected, olanzapine and clozapine are the drugs that are associated with the greatest risk, whereas quetiapine, risperidone, asenapine, and amisulpride show a moderate level of this undesired effect. Ziprasidone and lurasidone seem more tolerable on the metabolic profile; however, these drugs are relatively new, thus their therapeutic efficacy in comparison with other SGAs still needs to be determined. A reduced level of MetS has also been found for aripiprazole, an SGA with a different receptors profile compared to the other drugs (Roerig et al., 2011) (Reynolds & Kirk, 2010) (Kroeze et al., 2003) (Nasrallah & Newcomer, 2004).

1.6 SGAs and Dyslipidaemia

Dyslipidaemia is characterized by an abnormal lipid profile (e.g., elevated levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), and triglycerides and low levels of high-density

lipoprotein cholesterol (HDL-C)) (Cha & McIntyre, 2012). Altered lipid profiles associated with SGA use were initially considered as a consequence of adiposity caused by iatrogenic weight gain. However, further evidence indicates that changes in lipids may increase risks for insulin resistance and cardiovascular disease, independent of weight gain (D. D. Kim et al., 2019) (Correll et al., 2009). In a short-term trial investigated the effect of SGAs in paediatric patients, a significant increase in cholesterol and triglycerides was observed among patients receiving olanzapine and quetiapine, significant decreases in patients who received ziprasidone, and no change in those who received aripiprazole or risperidone compared to placebo (D. D. Kim et al., 2019).

The receptor-binding profiles that most closely correlate with SGA-associated dyslipidaemia are unclear, although peroxisome proliferator-activated receptors (PPARs), transcriptional regulators of lipid and carbohydrate metabolism, seem play a role (Arulmozhi et al., 2006). Various reports have proposed a link between the PPARs and diabetes, obesity, dyslipidaemia, and inflammation. A paper by Arulmozhi and collaborators (Arulmozhi et al., 2006) showed that the antidiabetic agents' rosiglitazone and glimepiride, both PPARa agonists, and the hypolipidemic agent fenofibrate, an agonist of PPARg, significantly decreases the level of triglycerides in mice treated with chlorpromazine, clozapine, and ziprasidone. Rosiglitazone, glimepiride, and fenofibrate have different PPAR-binding profiles and thus receptor activation can lead to an increase or decrease in target gene transcription. For instance, activation of the PPARa receptor results in the transcription of genes involved in fatty acid uptake and oxidation, inflammation, and vascular function, whereas PPARg binding upregulates genes involved in fatty acid uptake and storage, inflammation, and glucose homeostasis (Arulmozhi et al., 2006).

1.7 SGAs and Glucose Disturbances

The evidence for abnormal glucose regulation as a result of SGAs use indicates a heterogeneous pathophysiology and aetiology in the development of adverse events such as hyperglycaemia, diabetic ketoacidosis, new-onset diabetes mellitus and exacerbation of pre-existing diabetes mellitus, particularly in younger populations.

Recent studies have analysed the potential relationship between antipsychotic drug affinity for specific receptor subtypes and diabetes. For example, Matsui-Sakata and collaborators (36) reported that antipsychotic drug affinities for HTR2C, HR1, and MR1 are associated with an increased risk of diabetes.

Recently it has been proposed a serotonergic model for clozapine and olanzapine-induced diabetes. HTR1A antagonism, in a mouse model, decreases pancreatic b-cell responsiveness, thereby reducing insulin secretion and increasing serum glucose levels (J. Chen et al., 2017).

A study in healthy volunteers has shown that HTR2 antagonists can significantly decrease insulin sensitivity compared to placebo (P = 0.047), possibly through the suppression of HTR2A mediated glucose uptake in skeletal muscle (Matsui-Sakata et al., 2005). In addition, HTR2C knockout mice develop insulin resistance and impaired glucose tolerance and experience severe weight gain

Additionally, histamine and muscarinic receptors may also be associated with an increased risk of diabetes. The disruption of HR1 function may interfere with leptin-mediated appetite suppression, an effect that could lead to weight gain and insulin resistance (Matsui-Sakata et al., 2005). Moreover, antipsychotic drug affinity for muscarinic MR3 seems to be a significant predictor of the development of diabetes. This finding is supported by the fact that MR3 is highly expressed by pancreatic b-cells, where it might play a role in regulating glucose-dependent acetylcholine modulation of insulin secretion. Therefore, it may be possible that MR3 antagonism of SGAs is a precipitating factor in individuals predisposed to the development of diabetes (Silvestre & Prous, 2005).

1.8 SGAs and Prolactin elevation

Prolactin elevation has been observed among children and adolescents exposed to antipsychotics. Amenorrhea or galactorrhoea in females and gynecomastia in males are indications of elevated prolactin. The management of SGA-induced hyperprolactinemia may involve dose reduction and/or switching, use of a dopamine agonist (i.e., bromocriptine) or concomitant oral contraceptive use (for females) to eliminate symptoms related to estrogenic deficiency.

Elevations in serum prolactin have been hypothesized as a result of DRD2 binding. The impact of elevated prolactin on growth and development, as well as bone mineral density, remains unknown. (Panagiotopoulos et al., 2010) (Thesis & Maastricht, 2022). It is known that chronic elevations in prolactin is associated with osteoporosis. The risk of increased prolactin varies depending on the antipsychotic; risperidone seems to have the highest risk among SGAs (Thesis & Maastricht, 2022). In some studies, among children taking risperidone, prolactin levels peaked in short time (about 1-2 months) and remained elevated in the long term (6 months and 22 months). Conversely, other SGAs such as aripiprazole significantly decreases prolactin levels compared to placebo (Thesis & Maastricht, 2022) (Gupta et al., 2017).

1.9 SGAs Cardiovascular Effects

Antipsychotics may also cause cardiovascular side effects in exposed youth.

The risk of developing hypertension during antipsychotic treatment is reportedly higher among adolescents without correlation to a particular antipsychotic. One randomized controlled trial

described an increase in blood pressure by 4 mmHg among youth treated with both risperidone, and olanzapine (Waszak et al., 2019).

Tachycardia has also been reported (Waszak et al., 2019). The development of sinus tachycardia is most common and marked with clozapine with 17-33% of patients affected (Nilsson et al., 2017). This effect seems to be dose dependent (Hattori et al., 2018) and usually occurs transiently during dose titration, particularly if this is rapid. SGA-induced tachycardia is related to their antagonistic effects on receptors of the autonomic nervous system, such as MR1 and. ADRA1A. Antagonism of the former reduces vagal tone and of the latter reduces sympathetic activity and vascular tone, therefore leading to a reflex tachycardia (Hattori et al., 2018).

Antipsychotic medications prolong the QT interval, one of the most common reasons for referral to cardiology from psychiatry departments. This effect occurs because of their block of inwardly rectifying potassium channels (Kir) on the myocardial cell membrane, resulting in a reduction in this repolarising current, continuation of the action potential that is seen on the surface ECG as prolongation of the QT interval (Kongsamut et al., 2002). The increased duration of the action potential enhances the risk of developing ventricular arrhythmias, that in people taking SGAs has been estimated to be between 1.5 and 2.5 times higher compared with people with schizophrenia not taking antipsychotics (47). The increase in risk appears to be dose dependent (Ray et al., 2009), correlates with the QTc-prolonging properties of the drug and is highest when medication has been recently started (Ray et al., 2009) (Wu et al., 2015)

1.10 Other Risks associated to the use of SGAs in Youth

Sedation/Somnolence

Both FGAs and SGAs may cause sedation and somnolence. Patients prescribed SGA treatment often report the occurrence of these phenomena, particularly in situations requiring polypharmacotherapy. These effects are correlated with the high binding affinity for the HR1, that is also associated to changes in sleep phase duration (D. D. Miller, 2004). This alteration led to reduced wakefulness, attention, mental acuity, and overall cognitive function (D. D. Miller, 2004) (J. M. Kane & Sharif, 2008). Patients with psychiatric disorders frequently experience sleep disturbances (e.g., insomnia, hypersomnia, night terrors) and, although sedation/somnolence may help patients displaying acute psychotic features, it becomes problematic at later stages of disease progression (J. M. Kane & Sharif, 2008).

It has been demonstrated that SGAs mitigate acute agitation/aggression in patients with autistic disorder (D. D. Miller, 2004), dementia and schizophrenia (J. M. Kane & Sharif, 2008). This therapeutic effect may be an epiphenomenon of sedation/somnolence, which can be a barrier to

treatment acceptance for many individuals. Nevertheless, some SGAs (e.g., aripiprazole) cause less severe sedative effects than FGAs (e.g., chlorpromazine) (D. D. Miller, 2004).

Evidence indicates that treatment-naive patients experience greater sedation/somnolence severity, that often has been found to resolve over time. For most patients, however, persisting sedation/somnolence is an undesirable adverse event and should be avoided and/or treated by SGA dose reduction or by switching drug (D. D. Miller, 2004).

A recent comparison of paediatric and adult safety trials for antipsychotics submitted to FDA demonstrated that sedation is major side effect associated to the use of SGAs in children and the incidence of sedation is higher in paediatric patients than in adults (Liu et al., 2019).

1.11 Neutropenia and Agranulocytosis

Beyond the side effects discussed above, other potentially rare serious side effects to SGAs, such as agranulocytosis and neutropenia, have been reported. Except for clozapine, the decrease in white blood cells is generally not clinically significant with antipsychotics (Oh et al., 2020). A retrospective chart review carried out for clozapine-treated paediatric patients hospitalized at the National Institute of Mental Health (NIMH) between 1990 and 2011, showed that mild neutropenia was observed in 31% patients and moderate neutropenia in 20% patients (Maher et al., 2013). In general, the rate of neutropenia in children and adolescents treated with clozapine is considerably higher than in the adult population (Maher et al., 2013).

1.12 Neuromotor Adverse Effect - Extrapyramidal side effects.

Children and adolescents are more likely to experience extrapyramidal side effects (EPS) associated with both FGAs and SGAs than adults (T. et al., 2011). These are classified into acute syndromes, such as akathisia, dystonia, and Parkinsonism as well as tardive syndromes (i.e., tardive dyskinesia). A double-blind, randomized paediatric study directly compared EPS rates with an FGA (i.e., haloperidol) and SGAs (i.e., risperidone and olanzapine) (T. et al., 2011). Results suggest that children and adolescents are at risk for EPSs not only with haloperidol (67%) but also with SGAs (olanzapine (56%) and risperidone (53%)), at least at doses required to control psychosis but the severity was greater with haloperidol. As in adults, clozapine (Rummel-Kluge et al., 2012) and quetiapine (Divac et al., 2014) appear to be associated with relatively low EPS rates in paediatric patients; more data are needed for ziprasidone and aripiprazole. In a recent double-blind, placebo-controlled study of aripiprazole in adolescents with schizophrenia, EPSs occurred in 18% of patients (Rummel-Kluge et al., 2012).

Akathisia in children and adolescents, it can be difficult to properly diagnose akathisia because the symptom overlaps with psychomotor agitation due to psychosis, mania, and anxiety. In addition, patients experiencing akathisia may present difficulty falling asleep and this can be mistaken for the attention-deficit/ hyperactivity disorder. In one study (T. et al., 2011), olanzapine was associated with akathisia in 12.5% of paediatric patients. In a retrospective chart review evaluating the effectiveness and tolerability of aripiprazole for the treatment of 30 children and adolescents (mean age 13.3 years, range 5–19) with bipolar disorders, akathisia was recorded in as many as 23% of patients (Barzman et al., 2005). This effect is dose-dependent and variable depending on the starting dose of the drug.

Withdrawal Dyskinesia and Tardive Dyskinesia. Rates of withdrawal dyskinesia appear to be lower with SGAs compared with FGAs (Campbell et al., 1997), although a switch from an antipsychotic with strong DRD2 affinity (e.g., risperidone, aripiprazole) to one with less potent affinity (e.g., quetiapine) may predispose to withdrawal dyskinesia. In one study (Malone et al., 2002), 2 of 13 children (15.4%) developed mild, reversible withdrawal dyskinesia after 7 months of risperidone treatment.

Tardive dyskinesia usually appears after years of antipsychotic use and seems to be related to the total lifetime medication dose. Pooled data from long-term studies showed significantly reduced rates of tardive dyskinesia with SGAs compared with haloperidol. Results for paediatric patients are very poor because of the difficult to design a study to detect specifically tardive dyskinesia, the low dosages of the drugs, and the briefness of the exposure (Karaş et al., 2016).

Chapter 2
RISPERIDONE AND ARIPIPRAZOLE IN CHILDREN AND ADOLESCENT

Risperidone and aripiprazole are the most widely used SGAs in paediatric patients s. In this chapter we describe the main characteristics of the two drugs (pharmacodynamics, pharmacokinetics, pharmacogenetics, drug interactions and adverse reaction).

2.1 Risperidone

Risperidone is an antipsychotic agent with a benzoxazole chemical structure that has potent DRD2, 5-HT2A, and ADRA1A antagonism (Gardner et al., 2005).

On October 6, 2006, FDA announced the approval of risperidone, as the first drug to treat irritability and aggression in children with autism. On August 22, 2007, the FDA-approved indications for risperidone were expanded to include the treatment of bipolar disorder in children of 10 years of age and older and schizophrenia in patients of 13 years of age and older.

Risperidone is atypical at lower doses but can become more “conventional” at high doses, with EPS that can occur if the dose is too high (Bishop & Pavuluri, 2008).

2.1.1 Mechanism of Action

As for other antipsychotics, the exact mechanism of action for risperidone is not fully understood.

Schizophrenia and various mood disorders are thought to be caused by an excess of DRD2 and HTR2A activity, resulting in overactivity of central mesolimbic pathways and mesocortical pathways, respectively. Risperidone appears to reduce this overactivity through inhibition of DRD2 and HTR2A in the brain. DRD2s are inhibited by risperidone, therefore reducing dopaminergic neurotransmission, and decreasing positive symptoms of schizophrenia, such as hallucinations and delusions (64). Risperidone binds transiently and with loose affinity to the DRD2, with an ideal receptor occupancy of 60-70% for optimal effect (Fenton & Scott, 2005). Rapid dissociation of risperidone from the DRD2 contributes to decreased risk of extrapyramidal symptoms (EPS). A higher occupancy of DRD2 increases the risk of EPS and is therefore to be avoided (Sakhamuri et al., 2021).

Risperidone binds with a very high affinity to HTR2A and carries activity at several off-targets which may be responsible for some of its undesirable effects (Clarke et al., 2013) (Kozielska et al., 2012).

Risperidone also demonstrates high affinity for ADRA1A and ADRA2A and HR1 and moderate affinity for serotonin HTR2C, HTR1A, DRD3, and weak affinity for DRD1(Mirabzadeh et al., 2014).

2.1.2 Pharmacokinetic

After oral administration, risperidone is completely absorbed, reaching maximum plasmatic concentrations within one to two hours. Risperidone is rapidly distributed, with a volume of distribution of 1 to 2 L/kg. It is extensively metabolized via hydroxylation by cytochrome P450 isoform CYP2D6 to 9- hydroxy risperidone (9-OH-R), an active metabolite. Risperidone and 9-OH-R are ~88% and ~77% protein-bound in human plasma, respectively; they bind to both serum albumin and alpha-1-acid glycoprotein (Yoo et al., 2012).

Hydroxylation is dependent on debrisoquine 4-hydroxylase and metabolism is sensitive to genetic polymorphisms in debrisoquine 4-hydroxylase (Yoo et al., 2012). The half-life of risperidone and 9-idrossi-risperidone are 3 and 24 hours respectively.

2.1.3 Pharmacokinetics of risperidone: genetic background & clinical relevance

One of the main factors that may contribute to interindividual differences in drug response and toxicity is the variability in the steady-state plasma concentrations of the drug and/or its metabolites as a result of differences in drug metabolism.

The pharmacologic activity of risperidone is expressed as the sum of the plasma concentrations of risperidone and 9-OH-R. The plasma concentration of the active moiety correlates with drug efficacy and the presence of adverse drug reactions (ADRs).

Regarding risperidone biotransformation, the metabolic enzymes CYP2D6 and CYP3A are crucial.

The gene encoding for CYP2D6 is highly polymorphic: to date more than 100 allelic variants have been identified, including complete deletion and duplications of the gene (Fleeman et al., 2011). Deviations in the number and type of allelic variants as well as gene copy number yield four CYP2D6-predicted metabolic phenotypes: ultra-rapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer, and poor metabolizer (PM) (Fleeman et al., 2011). Compared with EMs – i.e., carriers of at least one normally functional allele –PM individuals carry two non-functional alleles and have a greatly reduced capacity to eliminate CYP2D6 drug substrates. Other genetically determined phenotypes are the IM phenotype, which results from the presence of reduced-function alleles, and the UM phenotype, resulting from CYP2D6 gene duplications of functional alleles. EM and IM phenotypes are the most common. EM comprise 43–67% of populations and IMs comprise an additional 10–44% . PM and UM phenotypes are less common at approximately 1–20 and 1–10%, respectively (Puangpetch et al., 2016) (M. Kane, 2021), and vary significantly according to ethnicity.

As risperidone is primarily metabolized by CYP2D6 (M. Kane, 2021), which can therefore affect drug levels in both youth and adults (Bork et al., 1999), different phenotypes may have significant clinical importance concerning adverse side effects and drug effectiveness. While the importance of CYP2D6 genotype continues to be discussed for adult patients, there is little systematic information available for children and adolescents, who have in treatment with risperidone drug (Fleeman et al., 2011). A recent retrospective cohort study assessed the association between CYP2D6 status and the risk for adverse events in paediatric patients exposed to risperidone for at least 4 weeks. The authors demonstrated that children who are CYP2D6 PMs or IMs have increased incidence of ADRs during risperidone treatment. These results are consistent with findings reported by other paediatric studies. In 2014, Youngster and collaborators performed an observational study of 40 paediatric patients with autism spectrum disorder treated with risperidone which showed an increase in ADRs such as weight gain and tardive dyskinesia in CYP2D6 PMs but no ADRs in the UMs (Youngster et al., 2014). Prolactin production also seems to be correlated with the rate of risperidone metabolism. In a recent study of 25 children (5to 15 years of age) receiving risperidone who were genotyped for CYP2D6 polymorphisms, those with the ultrarapid metabolizer phenotype had the highest concentrations of 9-OH-R and the highest prolactin-levels (Youngster et al., 2014).

In addition to CYP2D6, also the isoforms CYP3A4 and CYP3A5 are involved in risperidone metabolism. Patients non-expressing CYP3A5 exhibited higher plasma concentrations of both risperidone and 9-OH-R (Kang et al., 2009). However, different studies did not find differences in concentrations of risperidone and 9-OH-R regarding the CYP3A phenotype (Vandenberghe et al., 2015). Further studies are needed to estimate the influence of CYP3A phenotype on risperidone.

Risperidone has strong affinity for the P-glycoprotein (P-gp) drug transporter, encoded by ABCB1. This transporter regulates drug bioavailability by controlling the intestinal drug absorption, the renal excretion, and the transport across the blood–brain barrier. Among the over 12 000 variants identified in the ABCB1 gene region (Yoo et al., 2012) , three single nucleotide polymorphisms (SNPs) (rs1128503 [1236C>T], rs2032582 [2677G>T], and rs1045642 [3435C>T]) are the most studied variants, and results are inconclusive regarding SNP-phenotype associations. Some studies suggested that the phenotype of these silent SNPs may result from protein folding and stability rather than from changes in gene expression, and the effect of the structural alterations is expected to be more substrate specific. In this regard, true relationships between ABCB1 genotype and pharmacokinetics of antipsychotic agents, as well as clinical outcomes, are unclear. The gene that controls Pgp is in the same chromosome as the *CYP3A* gene (chromosome 7); this makes understanding the effect of the P-gp genotype more difficult.

Preliminary evidence suggests that patients who have the rare allele of those three SNPs may have better symptom response outcomes, higher risks of adverse events (olanzapine, risperidone), and higher plasma levels. However, as results across studies are mixed, the clinical utility of examining these variants is still uncertain (Yoo et al., 2012).

2.1.4 Drug-drug interactions

The significance of cytochrome enzyme-related pharmacokinetic interactions for risperidone has been widely investigated in adult patients (Spina & De Leon, 2007). The metabolism of several medicines, including b-blockers and antidepressants, is regulated by CYP2D6, therefore theoretically, drug interactions might happen when risperidone is used with other common CYP2D6 substrates.

Multiple medication use raises the risk of clinically significant drug interactions, necessitating dose adjustments for SGAs.

Fluoxetine, paroxetine, thioridazine, and levomepromazine and bupropion are the main CYP2D6 inhibitors, enhanced both the plasma levels and the active moiety of risperidone.

Administration of CYP2D6 inhibitors, such as paroxetine or bupropion to adult patients receiving risperidone, may invert the R:9-OH-R ratio and increase risperidone's risk of ADRs, such as cardiac effects (Spina & De Leon, 2007)(D'Arrigo et al., 2005). In patients stabilized on risperidone therapy who were concomitantly given the CYP2D6 inhibitor paroxetine, a significant increase in the serum level of risperidone but not of 9-OH-R, was found; only one patient had side effects (Parkinsonian symptoms) that might be due to the increase of risperidone plasma concentrations (Spina & De Leon, 2007).

Because of its antagonist activity at dopaminergic receptors, risperidone may antagonize the effects of levodopa or other dopamine agonists. Concomitant administration of risperidone and valproate may result in up to a 20% increase in serum valproate concentrations.

2.1.5 Pharmacogenetics of clinical response to risperidone: genetic variability in pharmacodynamics

Patients with identical plasma and tissue concentrations of risperidone may still vary extensively in their responses. Indeed, genetic variants in the molecules that are drug directly targets may be linked to difference in drug's response. Thus far, mostly DRD and HTR have been studied as predictors of the response to risperidone.

DRD2 genes that have been associated with DRD2 protein density and/or function have been studied in risperidone therapy. The DRD2 rs1801028 (p. Ser311Cys) has been significantly associated with

risperidone efficacy on the treatment of negative symptoms of schizophrenia. In addition, being heterozygous for the rs1801028 variant in DRD2 has been associated with a better response to risperidone compared to homozygous (Lane et al., 2004).

Variants in the DRD2 gene (rs1799978, rs1800497) and in the v-akt murine thymoma viral oncogene homolog 1 (AKT1) gene (rs3803300, rs2494732) are significant predictors of treatment response to risperidone (Zakharyan et al., 2020).

The DRD2 rs4436578-C homozygous genotype is also found to be associated with a significantly increased risk of body weight gain induced by long-term SGA treatment including risperidone in patients with schizophrenia (Paderina et al., 2022). The DRD3 gene variant rs167771 achieved a significant association with risperidone-induced extrapyramidal symptoms (Gassó et al., 2009).

An *in vitro* pharmacogenetic study showed that several variants in the HTR2A gene are associated with changes in the potency of four SGAs (aripiprazole, clozapine, quetiapine, and risperidone) at the cellular level (Grunina et al., 2020). The HTR2C promoter -759C/T polymorphism has also been associated with improvement in the positive and negative syndrome Scale (PANSS) response to risperidone (Reynolds et al., 2005), and with treatment duration and efficacy.

2.1.6 Adverse Effects

In a systematic review and meta-analysis by Pringsheim and collaborators (Pringsheim et al., 2011), mean weight gain was greater in young patients taking risperidone than placebo, with a mean difference of about 1.72 kg in short trials (3–10 weeks) and 1.95 kg in longer ones (6 months).

Risperidone can also increase serum prolactin levels, leading to suppression of the hypothalamic gonadotropin-releasing hormone. As a result, patients may develop galactorrhoea, amenorrhea, gynecomastia, or impotence. Long-standing increases in prolactin release can also lead to decreased bone density and could potentially affect growth. Although less common than with FGAs, there have been reports of neuroleptic malignant syndrome (NMS) and tardive dyskinesia with risperidone use. The risk for these reactions increases as the dose of risperidone is enhanced and receptor-site specificity is lost. (91). Other rare, but serious, adverse effects associated with risperidone include orthostatic hypotension, seizures, depression, arrhythmias, and hypersensitivity reactions (Fenton & Scott, 2005).

2.1.7 Dosage and administration

The recommended starting dose for risperidone in children with autism is 0.25 mg/day for children 15 to 19 kg and 0.5 mg/day for children > 20 kg. Therapy is typically initiated as a single daily dose in the morning or evening. The dose may be increased by 0.25 to 0.5 mg at a minimum interval of

every 2 weeks. The effective dose range provided by the manufacturer is 0.5 to 3 mg/day. In patients who develop somnolence with once-daily dosing, the dose may be divided and administered twice daily. Risperidone doses should be reduced in patients with underlying renal or hepatic disease (Pesaturo, 2009).

2.2 Aripiprazole

Aripiprazole is an antipsychotic drug approved by the FDA for schizophrenia in 2002. The efficacy and safety of aripiprazole have been demonstrated in various psychotic diseases. Aripiprazole is indicated in the USA for treatment of schizophrenia and bipolar I disorder in paediatric patients and has been recently approved for the treatment of irritability associated with autistic disorder in children and adolescents (Di Sciascio & Riva, 2015).

2.2.1 Mechanism of Action

Aripiprazole shows a unique mechanism of action compared with other antipsychotic drugs, displaying partial agonist activity at DRD2, DRD3, and HTR1A, and acts also as a HTR2A and HTR7 antagonist (Kikuchi et al., 2021) (Shapiro et al., 2003). Aripiprazole is classified as a third-generation SGA because of its unique mechanism of action distinctive from that of other atypical antipsychotics (Kikuchi et al., 2021). Aripiprazole functions as a DRD2 partial agonist, theoretically reduces dopamine output when dopamine concentrations are high, thus improving positive symptoms, and increases dopamine output when dopamine levels are low with an improving of mood, negative and cognitive symptoms. This is the reason why aripiprazole is often called a dopamine system stabilizer (Kikuchi et al., 2021). Aripiprazole, which has higher affinity at DRD2 than endogenous dopamine but is less likely to induce EPS and elevate serum prolactin levels compared to other antipsychotics.
The partial agonism action on serotonin HTR1A improve anxiety and depression and reduce antipsychotic-derived EPS (Tuplin & Holahan, 2017). Aripiprazole acts also as a HTR2C partial antagonist. Comparing to other SGAs, it appears to regulate appetite with less effect on weight and an anti-obesity effect (Tuplin & Holahan, 2017).
Aripiprazole acts also as an antagonist, but with low affinity on H1R, ADRA1A (Kirino, 2012) and MR1 (Kirino, 2012).

2.2.2 Pharmacokinetic

Metabolism of aripiprazole is predominantly hepatic, mediated mostly by CYP3A4 and CYP2D6 (Kubo et al., 2007). Its only known active metabolite is dehydro-aripiprazole (D-aripiprazole);

although aripiprazole is the predominant drug moiety in systematic circulation, both aripiprazole and D-aripiprazole show similar pharmacological properties. At any given time, d-aripiprazole is approximately 40% of the drug available in plasma. The half-life of aripiprazole is 75 hours while the half-life of the active metabolite is 94 hours (Kubo et al., 2007).

2.2.3 Pharmacokinetics of aripiprazole: genetic background and drug interaction clinical relevance

The only existing pharmacogenetic guideline in aripiprazole treatment is for CYP2D6. The FDA recommend dose reduction for CYP2D6 PM (Swen et al., 2011) (FDA 2002 a, 2002).

The CYP3A4 is aripiprazole's secondary metabolizing enzyme. Patients should receive a reduced dose when concomitant CYP3A4 inhibitors are prescribed.

Due to similarity between CYP3A4, CYP3A5 isoform (J. R. Kim et al., 2008) could be involved in aripiprazole metabolism. CYP3A5 *3/*3 carriers do not express CYP3A5, therefore, higher aripiprazole concentrations could be expected (Lamba et al., 2012). However, results are still contradictory.

Another gene studied in aripiprazole pharmacokinetics is ABCB1 (Koller et al., 2020). Among the different mutations of ABCB1, carriers of the two mutations 2677T > G/A (rs2032582) and 3435T > C(rs1045642) presented lower plasma concentration (Soria-Chacartegui et al., 2021).

2.2.4 Pharmacogenetics of clinical response to aripiprazole: genetic variability in pharmacodynamics

The impact of polymorphisms on the pharmacodynamics of aripiprazole is not well supported by substantial evidence. The most studied variants are the DRD2 TaqIA polymorphism. The *A1 allele corresponds with lower density of DRD2 in the striatum (Jönsson et al., 1999). Some groups reported better aripiprazole response for *A1 allele carriers (Kwon et al., 2008). Furthermore, C/C homozygotes for rs6277 in DRD2 gene present poorer response to aripiprazole (Shen et al., 2009). Also, carriers of C allele in rs6277 and *A1 allele in Taq1A had poorer cognitive performance (Ramsay et al., 2015). Other reported genetic variants are in HTR2A. The different variants of this receptor present different binding affinities for aripiprazole (Banlaki et al., 2015). Subjects with the HTR2A rs6311 (1438G > A/T) and rs6313 (102T> C) polymorphisms showed poorer aripiprazole response for negative symptoms (Banlaki et al., 2015).

2.2.5 Adverse events

In a controlled trial for early-onset schizophrenia paediatric patients treated with aripiprazole or placebo, the majority of spontaneously reported adverse effects were EPS, akathisia, and somnolence. Mean changes in body weight from baseline were minimal (Hirsch & Pringsheim, 2016). Less information is available for other paediatric populations, although recent open-label studies in patients with tic disorders, aggression, and disruptive behaviour disorders confirm that this drug has less impact on the metabolic profile. Comparing with the most SGAs, aripiprazole induced minimal changes in weight, body mass index, glucose, or lipid metabolism (Tuplin & Holahan, 2017).

Aripiprazole tended to lower prolactin levels, even below baseline when used as a single drug.

Other most common adverse drug reactions (>10% patients) to aripiprazole are mostly headache, agitation, insomnia, anxiety, nausea and vomiting, light-headedness, and constipation (Hirsch & Pringsheim, 2016) especially at starting doses. Light-headedness seems to happen due to the antagonism on ADRA1A, as well as tachycardia, bradycardia, and syncope. Antagonism on MR1 causes anticholinergic effects, such as dry mouth and constipation. Antagonism on HTR appear also to be in involved in constipation, as serotonin participates in the activation of colonic smooth muscle contraction (Wang et al., 2016). Headache and somnolence are thought to be caused by the antagonism on HTR, HR1 and ADRA1A, however, the molecular mechanisms involved are not completely known (Fang et al., 2016).

2.2.6 Dosage and administration

The recommended starting dose of aripiprazole is 10 or 15 mg daily, preferably administered with meals. Aripiprazole is effective at dosages up to 30 mg/ day ; however, doses higher than 10 to 15 mg/day are not more effective (De Hert et al., 2008). Dosage increases should be made no earlier than two weeks after therapy begins; this is the time needed to achieve a steady state plasma concentration (Kirino, 2012) .

Chapter 3
MECHANISM UDERLYING METABOLIC EFFECTS OF SGA IN YOUTH

3.1 Metabolic syndrome

There is a lack of long-term clinical data to define metrics and risk thresholds predicting the development of MetS in children (Zimmet et al., 2007). In 2007, the International Diabetes Federation (IDF) developed consensus guidelines for the diagnosis of metabolic syndrome in children. The IDF guidelines use waist circumference plus two other risk factors as diagnostic criteria. Waist circumference has been shown to predict metabolic syndrome with similar accuracy to BMI when gender, age, and ethnic group have been considered (Zimmet et al., 2007). The IDF Guidelines criteria defined for three age groups: 6-9 years, 10-15 years, and 16 and older are reported in figure 5.

Age group (years)	Obesity* (WC)	Triglycerides	HDL-C	Blood pressure	Glucose (mmol/L) or known T2DM
6–<10	≥90th percentile	Metabolic syndrome cannot be diagnosed, but further measurements should be made if there is a family history of metabolic syndrome, T2DM, dyslipidemia, cardiovascular disease, hypertension and/or obesity.			
10–<16 Metabolic syndrome	≥90th percentile or adult cut-off if lower	≥1.7 mmol/L (≥150 mg/dL)	<1.03 mmol/L (<40 mg/dL)	Systolic ≥130/ diastolic ≥85 mm Hg	≥5.6 mmol/L (100 mg/dL) (If ≥5.6 mmol/L [or known T2DM] recommend an OGTT)
16+ Metabolic syndrome	Use existing IDF criteria for adults, ie: Central obesity (defined as waist circumference ≥ 94cm for Europid men and ≥ 80cm for Europid women, with ethnicity specific values for other groups*) plus any two of the following four factors: • raised triglycerides: ≥ 1.7mmol/L • reduced HDL-cholesterol: <1.03mmol/L (<40 mg/dL) in males and <1.29mmol/L (<50 mg/dL) in females, or specific treatment for these lipid abnormalities • raised blood pressure: systolic Bϑ =130 or diastolic Bϑ =85mm Hg, or treatment of previously diagnosed hypertension • impaired fasting glycemia (IFG): fasting plasma glucose (FPG) =5.6 mmol/L (≥100 mg/dL), or previously diagnosed type 2 diabetes				

Figure 5. The IDF consensus definition of metabolic syndrome in children and adolescents (Zimmet et al., 2007).

The SGAs-induced Met dysfunction is the consequence of a very complex and broad activity of these drugs on the central nervous system (CNS) and peripheral organs, especially by interfering with the activity of neurotransmitter receptors expressed in these tissues. In addition, if we consider how the hypothalamus regulates the peripheral organs by altering the concentrations of neuromodulators and hormones in the blood system, the role of the CNS and each organ in the genesis of the SGAs-induced MetS is difficult to establish.

Hypothalamus is the main sensor of the nutrient concentrations in the blood and controls glucose and lipid homeostasis acting on several organs including the liver, pancreas, adipose tissue, and skeletal muscle. Hypothalamus stimulates the autonomic nervous system increasing noradrenaline and adrenaline levels, glucagon secretion, production of glucose, and lipolysis, and it reduces insulin secretion leading to transitory blood glucose increase.

Most antipsychotics have an affinity for a broad range of neurotransmitter receptors in the CNS in addition to the DRD, some of which have been associated with metabolic side effects. In particular, the risk of weight gain and glucose intolerance has been linked to an affinity to CNS histaminergic H1R, HTR1, HTR2A, and HTR2C, and MR1 and MR3 (Waterson & Horvath, 2015) (Morton et al., 2006).

3.2 Regulation of Appetite by SGAs through Neurotransmitter Systems

3.2.1 The Serotonergic System

There are several lines of evidence pointing toward a role for HTR2C and possibly HTR1/2A in SGA-induced hyperphagia and weight gain (Camilleri, 2015) (G. D. Miller, 2019).

It has been shown that HTR2C and serotonin work together to stimulate the cleavage of pro-opiomelanocortin (POMC), and the inhibition of neuropeptide Y (NPY) and agouti-related peptide (AgRP) by serotonin inhibiting (α- Melanocyte Stimulating Hormone) α-MSH and resulting in satiety and thermogenesis.

It is well-known that olanzapine and clozapine show antagonism and have a high affinity for serotonin HTR2A and HTR2C. In particular, the two drugs reduce the expression of the anorexigenic peptide POMC and conversely increase the expression of the orexigenic peptide AgRP and NPY. Because of these changes in the hypothalamus, SGAs increase the sympathetic efflux in the periphery by affecting glucose homeostasis (Camilleri, 2015) (G. D. Miller, 2019). Loss of HTR2C has been demonstrated to alter feeding behaviour and trigger obesity in mice. The importance of the HTR2C in SGAs has been recently observed in a rodent model, where olanzapine-induced hyperphagia and

weight gain are diminished in mice lacking the receptor expression. Moreover, treatment with the HTR2C specific agonist lorcaserin suppressed hyperphagia and weight gain induced by olanzapine, suggesting pharmacological treatment with this drug as a valid strategy to counteract SGA-induced weight gain. Risperidone possesses antagonism of HTR2; a recent study by Wan and collaborators carried out in rodents demonstrated that the HTR2C-NPY pathways are involved in the stimulatory effects of risperidone on appetite and weight gain.

Ziprasidone and aripiprazole are both associated with little or no weight gain (Wan et al., 2020). It has been reported that the coadministration of olanzapine with ziprasidone or aripiprazole does not induce food intake, possibly due to the partial agonism effect on HTR1A that they have in common, but other mechanisms may also be involved.

3.2.2 The Histaminergic System

Some of the orexigenic effects of SGAs are probably mediated by their effects on histamine H1R- and H3-receptors (H3R) (Masaki et al., 2004). Central histaminergic activity is known to repress food intake, and it has been reported that the administration of H1- antagonists causes hyperphagia. Accordingly, mice lacking H1R show increased food intake, altered eating habits, and obesity (Masaki et al., 2004). Conversely, H3R antagonists have the opposite effect and cause hypophagia (Deng et al., 2010). Olanzapine-treated rats show a reduction in H1R expression levels in hypothalamus with an increase in body weight and food intake compared with rats treated with haloperidol or aripiprazole (Masaki et al., 2004). Furthermore, both olanzapine and clozapine have been reported to reduce H1R levels in the hypothalamus, with the stimulation of AMP-activated protein kinase (AMPK), leading to an increase in food intake and weight gain (Jafari et al., 2012). In addition, it has been reported that, by suppressing postsynaptic H1R, olanzapine activates pre-synaptic H3 auto-receptors, reducing histamine synthesis and secretion, and aggravating hyperphagia (Karaş et al., 2016). Also, clozapine acts on H3 auto-receptors (with moderate affinity) to block acetylcholine (ACh) and noradrenaline (NA) release, resulting in dysregulation of appetite

To summarize, direct antagonism of hypothalamic H1R by SGAs may stimulate appetite by acting on hypothalamus, or weight gain may be influenced by SGAs partly by H3R.

3.2.3 Dopaminergic System

Dopamine antagonism (or, in the case of aripiprazole, partial agonism) is the core property of all SGAs. The mesolimbic and mesocortical dopaminergic pathways originating from are particularly involved in hedonic feeding (Baik, 2021). Upon ingestion of foods, dopamine is released into the ventral tegmental area (VTA) of the brain, leading to the activation of the neural mechanisms

connecting the VTA to the nucleus accumbens (NA) by the midbrain. Researchers have recently shown that DRD1 and DRD2 are localized in POMC-positive neurons in the brain of rats and mice (Romanova et al., 2017). These findings support the hypothesis that dysregulation in feeding behaviour mainly depends on the dysregulation of dopamine levels as well as receptor activity that can be controlled by SGAs. In addition, DRD2 block can contribute to the deregulation of glucose and lipid metabolism and induce hyperprolactinemia with consequences in food regulation and metabolism.

3.2.4 Acetylcholine System

MRs are involved in appetite regulation. According to Yasuhara and collaborators (Yasuhara et al., 2007) the MR3 is especially critical in the control of appetite and metabolism at the hypothalamus level. MR3 activity involves a complex crosstalk between peripheral nervous system and CNS, with controlling metabolic hormones via the vagus nerve linked to the hyperphagic, diabetogenic, and weight gain risks associated with SGAs. Olanzapine and clozapine are MR3 antagonists, and both have been reported to increase MR3 binding density in the CNS, which results in increased weight and food intake (Yasuhara et al., 2007). This effect of SGAs is also supported by findings that cevimeline (an MR3 agonist) reduces body weight gain caused by olanzapine treatment (M. Han et al., 2022).

3.3 Neuroendocrine Signalling in SGA-induced appetite weight gain and metabolic deregulation

Metabolic effects associated with SGAs might result from direct changes to neuroendocrine signalling or occur secondary to weight gain. SGA effects on leptin, adiponectin, and ghrelin have been examined as potential mediators of SGA-related changes in energy homeostasis. These signalling molecules impact at various levels of energy balance including appetite and feeding, energy expenditure, and metabolic rate. Insulin and leptin modulate the expression of neuropeptides in the hypothalamus, which regulate feeding behaviour and are considered the most important agents in regulating weight gain and energy homeostasis.

3.3.1 Leptin

Leptin is part of the anorexigenic pathway in appetite regulation. Leptin was the first adipokine identified to facilitate a link between the adipose tissues and the hypothalamus. Increased fat mass

results in elevated secretion of leptin, with subsequent reduction in food intake and increase in energy expenditure (Benbaibeche et al., 2021).

A study published by Endomba and collaborators (Endomba et al., 2020) demonstrated that leptin acts on neurons of the lateral arcuate nucleus within the hypothalamus inhibiting the expression of NPY and AgRP and stimulating POMC. POMC is modified into α-MSH, which can then stimulate melanocortin receptors 3 (MC3R) and 4 (MC4R), suppressing food intake (Mukherjee et al., 2022). In different mouse models it has been shown that alterations of MC4R and decreased MC3R expression are associated with leptin resistance and obesity. When leptin resistance occurs, energy expenditure and food intake are unbalanced, thereby increasing appetite and body weight (Endomba et al., 2020).

Leptin resistance induced by SGAs has been suggested to cause body weight gain in psychotic patients (B. J. Kim et al., 2008). Some researchers have examined leptin levels in patients receiving various SGAs (mainly clozapine and olanzapine) (B. J. Kim et al., 2008) Notably, several reports suggest that leptin levels in patients with psychosis might be higher than in healthy controls and that they may remain high during SGA therapy (B. J. Kim et al., 2008). Olanzapine, clozapine, and quetiapine significantly increase leptin levels and show a correlation between leptin and BMI (Potvin et al., 2015).

3.3.2 Ghrelin and Adiponectin

Ghrelin is mainly produced by the stomach and stimulates appetite. However, ghrelin has also been found in other organs such as the pituitary, the lungs, the pancreas, the gall bladder, the esophagus, the colon, the liver, the spleen, the thyroid, the heart, and in the hypothalamus (arcuate nucleus (ARC)) (Date et al., 2000).

Considering the molecular mechanism of appetite regulation, it has been reported that in the ARC and the hindbrain, both orexigenic (NPY/AgRP) and anorexigenic (POMC) neurons are shown to be modulated by ghrelin-expressing neurons (Schaeffer et al., 2013). Furthermore, ghrelin is known to activate AMPK in the hypothalamus to regulate food intake (Schaeffer et al., 2013). During treatment with some SGAs, ghrelin has been reported to play a significant role in modulating appetite and energy homeostasis. Serum ghrelin levels in subjects treated with atypical SGAs for at least 1 year were found significantly higher than in controls. Two studies carried out by the same research group demonstrated a significant increase in total plasma ghrelin levels as well as active ghrelin after olanzapine and risperidone administration (Murashita et al., 2005). In rats it has been shown that olanzapine treatment elevates hypothalamic ghrelin receptor expression (Wittekind & Kluge, 2015) , supporting the concept that ghrelin signalling plays a role in SGA-induced obesity. Conversely, in

patients with schizophrenia treated with risperidone or olanzapine, it was found that circulating ghrelin levels did not increase, but rather decreased, and that there was also no significant difference between the effect of the two drugs (Wittekind & Kluge, 2015). The drop in ghrelin levels following SGA therapy may be due to obesity and excess energy intake. However, the reduction in ghrelin levels may also be due to stress, which is common among psychotic patients (Boiko et al., 2022).

Huang and collaborators reported that the HTR2C receptor is dimerized with ghrelin receptor type 1a (GHSR1a) in order to inhibit orexigenic signalling, while HTR2C antagonists reduce dimerization and increase GHSR1a-induced food consumption (Huang et al., 2018).

Adiponectin is an adipokine secreted by white adipose tissue (WAT) and widely distributed in circulation. Adiponectin promotes fatty acid oxidation and insulin sensitivity in peripheral tissues by activating AMPK. At central level adiponectin increases AMPK activity in the ARC through the Adiponectin receptor 1 (AdipoR1), causing an increase in food intake (Kubota et al., 2007) .

SGAs can alter adiponectin levels. In a meta-analysis of 2015 (Bartoli et al., 2015), it has been demonstrated that people treated with clozapine and olanzapine display lower adiponectin levels than those taking risperidone. The lower adiponectin levels found in SGA treated patients indicate reduced insulin signalling and are normally associated with metabolic disturbances. The mechanisms by which SGAs might influence adiponectin levels have not been identified.

In conclusion, the dysregulation of appetite and weight gain associated with SGAs appears to be associated with altered serum ghrelin and adiponectin levels or their signalling. Further research is needed to clarify the causality in this interplay and to explain the inconsistencies observed between the studies.

3.4 Peripheral Mechanisms of SGA-Induced Metabolic Disturbances

Increasing evidence suggests that SGAs act on their molecular targets such as histamine, serotonin, and dopamine receptors, in peripheral organs critical for metabolic control, including the liver, pancreas, and adipose tissue (Mukherjee et al., 2022).

3.4.1 SGAs and Liver

Liver is the main location of glucose production, and it has a key role in the regulation of systemic glucose and lipid fluxes during feeding and fasting. Hormones secreted by the pancreas, such as insulin and glucagon, pass through the liver before entering the systemic circulation. The SGA-induced increase of glucagon targets the liver, and the excessive production of glucose are responsible for the development of Type 2 diabetes (T2D), even at early stages. Clozapine and olanzapine may

induce an increase in glucagon levels even when peripheral glucose levels are high (Boyda et al., 2022).

A confirmation of SGAs-induced glucagon increase for the hyperglycaemic effect is based on data demonstrating that olanzapine-induced increase of blood glucose levels was found abolished in glucagon receptor KO mice (Castellani et al., 2017). Besides glucagon increase, the diabetogenic effect induced by SGAs including clozapine and olanzapine is also mediated by hepatic insulin resistance, which contributes to an abnormal increase in hepatic glucose output (Smith et al., 2014). Notably, this effect was not observed with risperidone (Hui Fang et al., 2018).

Concerning hepatic metabolism, AMPK is known as key player in glucose homeostasis because its activation decreases blood glucose levels, mostly by inhibiting gluconeogenesis. SGAs significantly decrease AMPK activity thus altering glucose metabolism (Tarasiuk et al., 2022). Furthermore, since activated AMPK also regulates lipid metabolism by inhibiting lipogenesis and increasing fatty acid oxidation, it is reasonable to assume that SGAs increase hepatic lipogenesis through AMPK inhibition, which contributes to liver fat accumulation (146). AMPK activation inhibits the mechanistic target of rapamycin complex 1 (mTORc1) function; therefore SGAs indirectly stimulate mTOR signalling increasing the expression of the transcriptional activation of sterol-regulatory element-binding proteins 1c (SREBP-1c). SREBPs play a fundamental role in controlling a variety of lipid biosynthetic pathways. SGA-induced hepatic overexpression of SREBP-1c is relevant for lipid accumulation and liver steatosis, that have been demonstrated for clozapine, olanzapine, and risperidone. Aripiprazole and haloperidol did not induce the same effect (Cai et al., 2015).

SREBPs activity seems to be controlled by the pathways of different receptors present in the liver including, HTR2 and HR1, whose expression is increased in the liver of patients chronically exposed to SGAs (Xu & Zhuang, 2019). In addition, it has been demonstrated that SGAs decrease the transcriptional activity of PPAR, another critical regulator of lipolysis and fatty acid oxidation in the liver but also in the adipose tissue (Cai et al., 2015).

3.4.2 SGAs and Pancreatic β-Cells

SGAs treatment is associated with T2D, an undesired effect independent of weight gain. Since hyperglycaemia and peripheral insulin resistance are common side effects induced by SGAs, compensatory hyperinsulinemia should be expected in any case. A recent clinical study demonstrated that patients treated with clozapine or olanzapine showed hyperinsulinemia; however, it is not clear whether this was a direct consequence of β cell stimulation by SGAs or a compensatory mechanism to insulin resistance [148]. DRD2, DRD3, HTR2A/2C and HTR1A expressed in the beta cells might be partially responsible to the mechanism of SGAs-induced hyperinsulinemia. In normal conditions,

peripheral dopamine and serotonin generally slightly inhibit insulin secretion. Contrariwise, DRD2 antagonists like SGAs moderately raise insulin secretion (Rubi et al., 2005). Therefore, it is possible that the combination of dopaminergic and serotonergic antagonism might contribute to SGAs-induced insulin hypersecretion. In addition, the antimuscarinic properties of some SGAs, such as clozapine and olanzapine, affect the parasympathetic control of β-cell insulin secretion. Regarding this aspect, the effect of SGAs is controversial because besides blocking muscarinic receptors, clozapine and olanzapine appear to increase the parasympathetic output probably leading to activation of the vagal system with consequent stimulation of the β-cells (Sasaki et al., 2006).

3.4.3 SGAs and Adipose Tissue

Studies in both animal and human have demonstrated that treatment with SGAs increases lipogenesis, visceral fat reserves, free fatty acids (FFA) circulation, and pre-adipocyte differentiation (Gonçalves et al., 2015) (Rojo et al., 2015). Olanzapine and clozapine induced increased lipogenesis in adipocytes from rats; this effect seems to be promoted by up-regulating SREBP1c (Pillinger et al., 2020).

SGAs differ from other drugs because of their capability of inducing hypertriglyceridemia. Clozapine and olanzapine have the highest predisposition to increase triglycerides levels, while quetiapine and risperidone are associated with moderate risk, and ziprasidone, lurasidone, and aripiprazole with a minimal risk (Jassim et al., 2012). SGAs like olanzapine and clozapine not only have these effects but also raise plasma FFA levels. Increased FFAs reduce insulin's capacity to regulate hepatic glucose synthesis and promote skeletal muscle glucose uptake (Jassim et al., 2012). Hepatocytes, myocytes, and adipocytes remove FFAs from the plasma and convert them into activated fatty acids, which are then metabolized via oxidation or conserved via lipogenesis for later use (Gao et al., 2004) (Schenk et al., 2008).

Besides metabolic alterations, SGAs can stimulate the differentiation of preadipocytes into adipocytes, particularly in those located in white visceral adipose tissue, therefore contributing to adipose tissue mass. Olanzapine and clozapine upregulated the expression of transcription factors essential for adipocyte differentiation , such as PPAR-γ, CCAAT-enhancer binding protein β (C/EBPβ), and adipocyte determination and differentiation factor 1/SREBP1C (ADD1/SREBP-1C). Finally, the balance between the proliferation and differentiation of preadipocytes is regulated by intracellular oxidative stress and SGAs increase mitochondrial reactive oxygen species (ROS) production and oxidative stress levels (Gonçalves et al., 2015) (Rojo et al., 2015).

Due to increased adiposity, a rise in leptin levels generally occurs after SGA administration, which in the long term can lead to leptin resistance in the hypothalamic centres, thereby altering appetite regulation (Barateiro et al., 2017). Recently, it has been demonstrated in 140 subjects that low

adiponectin and high leptin levels in adipose tissues may contribute to increased oxidative stress and inflammation, which can be a marker for the severity of MetS (Frühbeck et al., 2017). All these alterations indicate the relevance of adipose tissue in the development of metabolic dysfunction such as insulin resistance, overstimulation of beta-cells insulin secretion, and inflammation, which in the long-term could lead to T2D, obesity, and Mets.

3.5 Pharmacogenetics of antipsychotic-induced metabolic dysfunction

Genetic factors may play an important role in SGA-induced MEtS. Genome-wide association studies have found multiple genes associated with obesity in the general population (Yoshida & Müller, 2019).

Genes related to antipsychotic metabolism and plasma drug levels have been emphasized in the prevalence of side effects. Most SGA are metabolized by CYP450 isoenzymes (Lett et al., 2012). Pharmacokinetic studies have hypothesized that poor CYP450 activity could be associated with increased serum levels of antipsychotics that may lead to increased weight gain (Lett et al., 2012).

In addition to drug metabolizing enzymes and drug transporters, pharmacogenetic research has also focused on genes encoding therapeutic targets of antipsychotic pharmacotherapies.

A recent meta-analysis that included papers up until the end of 2016 examined all genetic variants considered relevant in association with antipsychotic-related weight gain. 13 SNPs from 9 genes (Adrenoceptor Alpha-2A [(ADRA2A], Adrenoceptor Beta 3 [(ADRB3], Brain-Derived Neurotrophic Factor [BDNF], DRD2, Guanine Nucleotide Binding Protein [GNB3], HTR2C, Insulin-induced gene 2 [INSIG2], MC4R, and Synaptosome associated protein, 25kDa [SNAP25]) were significantly associated with antipsychotic-related weight gain (P-values: ≤.05–.001). (160)

HTR influence central pathways affecting satiety and hunger. The rs3813929 promoter SNP (759C/T) of HTR2C gene is one of the most consistent markers associated with increased weight gain. Another variant in the HTR2C gene is the rs6318 (Cys23Ser) SNP that may disrupt a disulphide bridge, thus suggesting functional significance. 12 polymorphisms in four HTR genes (HTR1A, HTR2A, HTR2C, and HTR6) have been reported so far. A promoter variant in the HTR2C gene, rs518147, and the combined haplotype (c.1-11429(GT)n 13 repeat (13R), 759C/T and 401C > G) were associated with the risk of obesity and metabolic syndrome (Lett et al., 2012).

The dopamine system also appears to be associated metabolic dysregulation. Three of the SNPs in DRD2 showed significant associations with weight gain. For rs1799732, the deletion of C was associated with weight gain. For rs6275, the T-allele was the risk allele because TT homozygotes gained more weight than C carriers and CC homozygotes gained less weight than T carriers (Zhang et al., 2016).

It is possible that these variants of DRD2, may produce fewer receptors than normal, and the subsequent over-eating behaviour and weight gain are exacerbated using antipsychotics.

Two SNPs in ADRA2A and ADRB3 (rs1800544, rs1051312) were significantly associated with weight gain. The adrenergic system innervates adipose tissue, in which the brown adipocyte is involved in the production of heat (thermogenesis or fat burning) and has an inhibitory effect on lipolysis in the adipose tissue (Zhang et al., 2016).

Another gene that has moderate evidence for its association with MetS is MC4R. The AA homozygotes of rs489693 gained more weight than the C allele carriers. For INSIG2, BDNF, and SNAP25 one SNP of each of these 3 genes was significantly associated with MetS (rs17047764, rs6265, rs1051312 respectively) (Zhang et al., 2016) (Lett et al., 2012).

AIM OF THE STUDY

With this project, we intend to define the role of SNPs in genes related to the pharmacodynamics of SGA, expressed in peripheral tissues, to evaluate their involvement in the mechanisms responsible for weight change and metabolic ADRs. The main aims of this project are:

Aim 1: Evaluate the association of SNPs found in selected genes with the occurrence of changes in BMI -Z score and weight in a cohort of paediatric patients. This allows us to evaluate if the presence of specific variant alleles increases or decreases the risk of weight changes and metabolic adverse events, thus contributing to defining a model of personalized medicine based on the individual characteristics of each patient.

Aim 2: Examine the direct effect of SGA-induced weight gain at the peripheral level. To this end, we studied the SGA's direct effect in a peripheral tissue model, adipose tissue, by using a human adipocytic cell line.

Aim 3: Validate in the adipocyte cell line the impact of SNPs on differentiation. To this end, we have designed an in vitro model system in which we obtained the introduction of selected SNPs, present in our cohort of pediatric patients to examine their role in metabolic pathways.

MATERIALS AND METHODS

Study setting

In this observational study 209 paediatric patients affected by disruptive behavioural disturbances associated with several psychiatric conditions who are prescribed SGAs (risperidone, aripiprazole) have been enrolled by four neuropsychiatry units. At the first visit blood has been sampled for genetic analyses. Referred to the clinic, each patient underwent a medical evaluation that included age, gender, anthropometric measurements (weight, height, waist circumference), neuropsychiatric diagnoses (according to DSM-IV TR); information on drug therapy.

All data collected for each patient is kept in form anonymous, in a database specially developed for the study (MMN.it).

The database is available online for entry, consultation, and data entry from part all the members of the project.

Pharmacogenetic analysis

Genomic DNA (gDNA) was isolated from peripheral blood cells using a DNA extraction system (The EZ1 Advanced XL instrument, Qiagen) according to the manufacturer's instructions. DNA concentration and purity were evaluated by absorbance methodology using a Nanodrop 1000 Spectrophotometer V3.7 (Thermo Scientific). SNPs in pharmacodynamic genes were determined by real-time PCR using a panel of pre-designed TaqMan Probe (Thermo Scientific) according to the manufacturer's instructions. The amplification was performed with LightCycler 480 System (Roche Diagnostic, Mannheim, Germany). The analyzed polymorphisms are indicated in table 1.

For CYP2D6 genotype, DNA samples were performed using the INFINITI CYP450 2D6-BC Assay a BioFilmChip™ Microarray (Autogenomics), i.e., a film-based matrix for the retention of an oligonucleotide array, acquired and processed by the INFINITI analyzer (Autogenomics). This technology and associated software detects 16 CYP2D6 allelic variants including gene duplication and deletion: CYP450 2D6 *2 (2850C>T) *9 (2615_2617delAAG) *3 (2549delA) *10 (100C>T) *4 (1846G>A) *12 (124G>A) *5 (CYP2D6 deletion) *17 (1023C>T) *6 (1707delT) *29 (1659G>A) *7 (2935A>C) *41 (2988G>A) *8 (1758G>T) *XN (multiple CYP2D6) *14 (1758G>A) *2A(−1584C>G).

The combination of CYP2D6 alleles is used to determine a patient's diplotype. Each allele is assigned an activity value ranging from 0 to 1 (e.g., 0 for no function, 0.25 or 0.5 for decreased function, and 1 for normal function); If an allele contains multiple copies of a functional gene, the value is

multiplied by the number of copies present. Thus, the CYP2D6 activity score is the sum of the values assigned to each allele. The CYP2D6 activity score can be translated into a standardized phenotype classification system. (Figure 1) (Crews et al., 2021)

Phenotype[a]	Activity score range	Activity score/genotypes[b]	Examples of *CYP2D6* diplotypes[b]
CYP2D6 ultrarapid metabolizer	> 2.25	> 2.25	**1/*1xN, *1/*2xN, *2/*2xN*[c]
CYP2D6 normal metabolizer	1.25 ≤ x ≤ 2.25	1.25 1.5 1.75 2.0 2.25	**1/*10* **1/*41, *1/*9* **10/*41x3* **1/*1, *1/*2* **2x2/*10*
CYP2D6 intermediate metabolizer	0 < x < 1.25	0.25 0.5 0.75 1	**4/*10* **4/*41, *10/*10* **10/*41* **41/*41, *1/*5*
CYP2D6 poor metabolizer	0	0	**3/*4, *4/*4, *5/*5, *5/*6*
CYP2D6 indeterminate	n/a	An individual carrying one or two uncertain function alleles	**1/*22, *1/*25, *22/*25*

Figure 1. Assignment of predicted CYP2D6 phenotypes based on diplotypes. (Crews et al., 2021).

Gene	Variant	Genotype
ADRA2A	rs1800544 (c.-1252G>C)	GG GC CC
ADRB3	rs4994 (c.190T>C)	TT TC CC
DRD2	rs1799732 (c.-486_-485insC)	-/- -/C CC
DRD2	rs6275 (c.939T>C)	TT TC CC
DRD2	rs7131056 (c.-32+16024T>G)	TT TG GG
DRD2	rs1799978 (c.-585A>G)	AA AG GG
HTR2A	rs6313 (c.102C>T)	CC CT TT
HTR2C	rs3813929 (c.-759C>T)	CC CT TT
HTR2C	rs6318 (c.68G>C)	GG GC CC
HTR2C	rs518147 (c.-697C>G)	CC CG GG
HTR2C	rs1414334 (c.551-3008C>G)	CC CG GG
MCR4	rs17782313 (g.60183864T>C)	TT TC CC
MCR4	rs489693 (g.60215554C>A)	CC CA AA
DRD3	rs6280 (c.25G>A)	GG GA AA
HTR6	rs1805054 (c.267C>T)	CC CT TT

Table 1. Polymorphisms of genes coding for proteins involved in SGA pharmacodynamics.

Cell line and differentiation protocol

Human liposarcoma SW872 cells were purchased from the American Type Culture Collection (Rockville, MD. Cells were routinely maintained in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12) (Euroclone), supplemented with 10% foetal bovine serum (FBS) (Corning), 100 U/ml penicillin, 100μg/ml streptomycin (Euroclone), under a humidified 5% (vol/vol) CO_2 atmosphere at 37°C.

Pre-adipocyte were differentiated into adipocytes by using a differentiation medium (DM) composed of 1μM dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA), 0,1 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, Saint Louis, MO, USA), 30 μM bovine serum albumin (BSA)-bounded oleic acid/linoleic acid (Sigma-Aldrich, Saint Louis, MO, USA) and 10 μg/ml insulin (Sigma-Aldrich, Saint Louis, MO, USA) in DMEM/F12 with 10% FBS. (Fiorani et al., 2021)

Cells were plated in the appropriate plastic ware based according to the type of experiments to be performed and were allowed to grow for 2-3 days to reach confluence. Confluency provides the signals for the cell to arrest the growth and start the differentiation (time 0). The DM was changed every 48 h and the differentiation process was followed up to day 10. The analyses were performed at different time points (3, 6, 10 days) of SW872 cell differentiation.

Cell Viability Assay and treatment

The dose of risperidone (Sigma-Aldrich, Saint Louis, MO, USA) to be used in the differentiation experiment was selected based on the results of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay (Sigma-Aldrich, Saint Louis, MO, USA) performed in SW872 preadipocytes.

Briefly, 5,000 cells were seeded in a 96-well plate overnight and treated with gradient concentrations (0.01; 0.02; 0.10; 0.5; 1; 10; 50; 100 μM) of risperidone for 48 h. Then, 90 μl of PBS and 10 μl of MTT solution were added to each well and incubated for 1–4 h. The purple formazan crystals were dissolved in DMSO, and the absorbance was recorded using a Glomax Multi detection system plate reader (Promega, Madison, WI, USA) at a wavelength of 570 nm. The assay was carried out in triplicate.

To explore the effect of risperidone on the differentiation process, SW872 confluent undifferentiated cells (time 0) were treated with risperidone, at the final concentration of 1 μM. The drug was added every 24h and the differentiation process was followed up to day 6.

Oil Red O staining

For the Oil red O staining the medium was removed from each well, cells were twice rinsed in phosphate-buffered saline (PBS) and fixed in 4 % formaldehyde prepared in PBS for 15' at room temperature. Then the cells were washed twice in distilled water and stained in Oil Red O solution. This solution was prepared by dissolving 0,3 g of Oil Red O powder (Sigma-Aldrich, Saint Louis, MO, USA) with 1 ml of isopropanol 100% (Sigma-Aldrich, Saint Louis, MO, USA) and filtered with absorbent paper. The solution was mixed in a 1:3 ratio: one part 0.5 % solution of Oil Red O and three parts distilled water. The cells were incubated for 45 minutes with Oil Red O solution at room temperature and, after being washed twice in distilled water to remove the unbound dye, the cells were subjected to a cleaning treatment. (Koopman et al., 2001)

Samples were then pre-incubated for 30 min at room temperature with 5% bovine serum albumin (BSA; Life Technologies, Monza, Italy) and 10% of normal goat serum (Life Technologies, Monza, Italy) in PBS containing 0.1% Triton X-100. Subsequently, samples were stained for 1 hour at room temperature with phalloidin-Alexa Flour 488 (Sigma-Aldrich, Saint Louis, MO, USA) that binds to F-actin filaments (cytoskeleton detection). Finally, samples were cover-slipped in a ProLong Gold Antifade Mountant containg DAPI a blue-fluorescent DNA dye (Life Technologies, Monza, Italy). Images were acquired with a Zeiss LSM 710 confocal microscope (Carl Zeiss) and analyzed by the ImageJ software.

Quantitative Real Time-PCR (qPCR)

Total RNA from cells was extracted with the PureZol RNA Isolation Reagent (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. First-strand cDNA was generated from 800ng of total RNA using iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). A set of primer pairs (Eurofins Genomics, Milan, Italy) was designed to hybridize to unique regions of the appropriate gene sequence (Table 2). qPCR was performed using the SsoAdvanced Universal SYBR Green Supermix and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The fold change was determined relative to the control after normalization to internal standards (rpl32, GAPDH) using the formula $2-\Delta\Delta CT$.

Gene	Primer Sequence
RPL32	(fw) 5'-TTAAGCGTAACTGGCGGAAAC-3' (rev) 5'-AAACATTGTGAGCGATCTCGG-3'
GAPDH	(fw) 5'-TGAGGTGAATGAAGGGGT-3' (rev) 5'-GTGAAGGTGGGAGTCAAC-3'
CEBP delta	(fw) 5'-CAGCAACGACCCATACCTCA-3' (rev) 5'-TCTTTGCGCTCCTATTATGTCCC-3'
CEBP beta	(fw) 5'-GAGGAGAAACTTTAGCGAGTCAGA-3' (rev) 5'-GGGTGGCCGCTATTAGTGAG-3'
CEBP alpha	(fw) 5'-ACTTGGTGCGTCTAAGATGAGGG-3' (rev) 5'-CATTGGAGCGGTGAGTTTGC-3'
PPAR gamma	(fw) 5'-AGGCGAGGGCGATCTTG-3' (rev) 5'-CCCATCATTAAGGAATTCATGTCATA-3'
UCP1	(fw) 5'-TCTCTCAGGATCGGCCTCTA-3' (rev) 5'-CCGTGTAGCGAGGTTTGATT-3'
PRMD16	(fw) 5'-GTTCTGCGTGGATGCAAATCA-3' (rev) 5'-GGAGAGGTTCTGGTCATCGC-3'
PGC-1α	(fw) 5'-CCAAGTCGTTCACATCTAGTTCA-3' (rev) 5'--TCGAGTCTGTATGGAGTGACAT3'
HOXC9	(fw) 5'-CAGCAACGACCCATACCTCA-3' (rev) 5'-TCTTTGCGCTCCTATTATGTCCC-3'
HTR2C	(fw) 5'-TCTTAATGTCCCTAGCCATTGCT-3' (rev) 5'-CCSGCGATATAGCGCAGAGG-3'
DRD2	(fw) 5'-CAGCAACGACCCATACCTCA-3' (rev) 5'-TCTTTGCGCTCCTATTATGTCCC-3'

Table 2. Primers used in this study

Protein Isolation and Western Blotting

Cells were lysed for 10 min at 4° C in a lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 10 mM EGTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, extemporaneously supplemented with a cocktail of protease and phosphatase inhibitors (cOmplete and PhosSTOP, Roche Applied Science Mannheim, Germany). This step was followed by sonication and, after waiting 30 minutes to let the foam extinct, by the addition of SDS 1% in the final volume. Then, 10-minute centrifugation at 4°C at 10000 g was needed to remove residual precipitates. Equal amounts of proteins (40 µg/lane) were loaded on polyacrylamide precast gels (Criterion TGX Stain-free precast gels, Bio-Rad), along with 2-4 µg of a protein marker (KaleidoscopicTM, Bio-Rad). The voltage was set constant at 130 V for 1 hour. Stain-free gels allowed the immediate visualization of proteins, resulting in fluorescence when exposed to UV light. This short photoactivation was performed at ChemiDocMP Imaging SystemTM (Bio-Rad, Hercules, CA, USA). Proteins were then transferred onto a nitrocellulose membrane using a Trans-Blot Turbo SystemTM and Transfer packTM (Bio-Rad, Hercules, CA, USA). The transfer occurred in 7 min at 2.5 mA. Total proteins transferred onto the membrane were acquired at ChemiDocMP Imaging SystemTM and afterward stained with Red Ponceau for 1 minute, to check the transfer quality and to allow the cutting of the membrane. Blocking was performed for 1 hour with 5% milk in Tris-Buffered Saline supplemented with Tween 20 (TBST). Primary antibody

solutions (Table 3) were incubated overnight at 4°C and properly diluted according to the manufacturer's recommendation. After incubation with horseradish-peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) for 1 hour at room temperature, bands were visualized using the Clarity Western ECL substrate with the ChemiDoc MP imaging system. Bands were quantified for densitometry using the Image Lab software (Bio-Rad, Hercules, CA, USA).

Epitope	Product name (catalogue number)	Manufacturer	Dilution
HTR2C	HTR2C SR-2C (D-12)	SANTA CRUZ BIOTECHNOLOGY	D 1:100
DRD2	D2DR (B-10)	SANTA CRUZ BIOTECHNOLOGY	D 1:100

Table 3. Primary antibody used in this study

Immunofluorescence

SW872 cells were cultured in 120-mm coverslips, washed in PBS, and fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 10 min. Samples were then washed in PBS and pre-incubated for 30 min at room temperature with 5% bovine serum albumin (BSA; Life Technologies, Monza, Italy) and 10% of normal goat serum (Life Technologies, Monza, Italy) in PBS containing 0.1% Triton X-100. Subsequently, incubation of primary antibodies was performed overnight at 4°C in a humidified chamber with proper dilution in blocking solution (Table 4). The following day, cells were washed 3 times with PBS and stained for 1 h at room temperature with the appropriate Alexa Fluor secondary antibody with or without phalloidin-Alexa Fluor 488 Finally, samples were cover-slipped in a ProLong Gold Antifade Mountant with DAPI. Images were acquired with a Zeiss LSM 710 confocal microscope.

Epitope	Product name (catalogue number)	Manufacturer	Dilution
c-Myc	c-Myc (9E10) sc-40	SANTA CRUZ BIOTECHNOLOGY	D 1:50

Table 4. Primary antibody used in this study

Mitochondria Respiratory Rate

Mitochondria respiratory rates were measured into the O2K oxygraphy chambers (Oroboros Instruments, Innsbruck, Austria) at 37° C in the respiration medium MiR06 (0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2PO4 20 mM Hepes, 110 mM sucrose and 1 g/L bovine serum albumin fatty acid-free, 280 U/mL catalase (pH 7.1)). Basal respiration was observed when the signal of oxygen consumption was stable, while the leak respiration was induced by the addition of oligomycin (0,5 µM). Stepwise titration (0,5 µM each step) of the uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) induced the progressive release of the proton gradient until maximal respiration was achieved. The residual oxygen consumption was evaluated by blocking mitochondrial respiration with the addition of 0.5 µM rotenone and 2.5 µM antimycin A (AA), and this value was subtracted by each steady state.

ATP Production

ATP concentration was measured by the luciferin-luciferase assay. Briefly, cells were permeabilized with digitonin (10µg/106 cells) in buffer-A (150mM KCl, 25mM Tris-HCl, 2mM EDTA, 0.1% BSA, 10mM potassium phosphate and 0.1mM MgCl2 (pH 7.4)) at room temperature with gentle agitation. Permeabilised cells were plated in 96 wells (2 × 105 cells/well) and treated with a mix containing 2 mM malate, 1 mM pyruvate, 1 mM ADP, and buffer-B (0.2 mM luciferin and 5 µg/mL luciferase in 0.5M Tris-acetate (pH 7.75)). Oligomycin (2 µg/mL) was also added to detect glycolytic ATP in our samples. ATP was measured using a GloMax luminometer.

Generation of stably transfected clones

To create a specific mutation in DRD2 and HTR2C receptors, the cDNA of the two genes were subcloned into a neomycin-resistant pcDNA3.1(+) _myc tag – vector with a CMV promoter for expressing C-terminally Myc-tagged proteins and with ECOR I (al 5'); Not I (al 3') restriction sites. Neomycin (G418) resistance gene is fundamental for the selection of stable transfected cells. (GenScript, Piscataway, USA)

The HTR2C rs6318 and DRD2 rs6275 variants were achieved using site-directed mutagenesis (Genscript). The transfection was performed using TransIT-X2 Dynamic Delivery System (Mirus BIO Madison, WI, USA). Cells were seeded in 6 well plates at 40% confluence and transfected with 2.5 µg of the expression vectors or the pcDNA3.1(+) _EMPTY-vector or PLVX_GFP vector, when they reach ~80% confluent, according to the manufacturer's protocol.

The transfection efficiency was evaluated at 48h post-transfection by assessing GFP+ cells. To generate stably transfected cell, 48–hours post-transfection cells were seeded in a complete growth medium containing G418 500ug/ml (Euroclone, Pero, Italy). After 10–15 days, clones were picked and subcultured under the same selection conditions. To identify the positive clones the expression of the MYC tag was observed with a Zeiss LSM 710 confocal microscope (Carl Zeiss), after the staining with the appropriate primary and secondary fluorescent antibodies.

Transcript and protein expression of the receptors in selected clones were then checked by qRT-PCR and Western blot analysis, respectively.

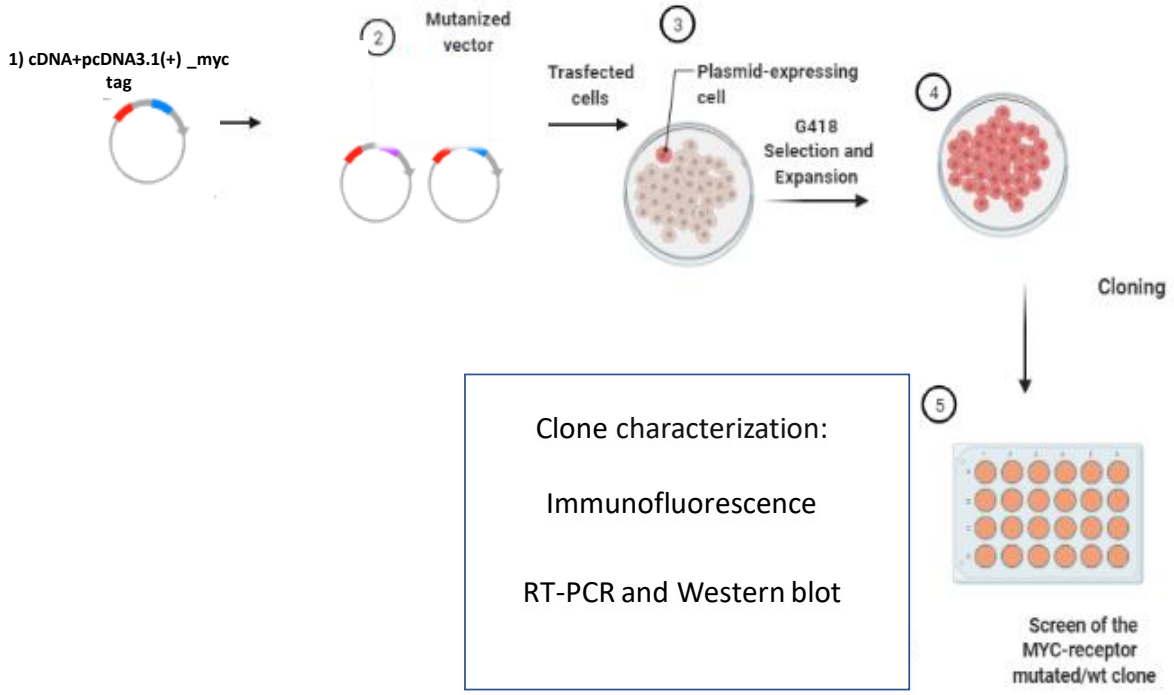

Figure 2. Schematic representation of transfection and selection protocol for HTR2C and DRD2 clones

Statistical analysis

To describe population, qualitative data were expressed as median (IQR), whereas qualitative were described as frequency and percentage.

BMI values were transformed into BMI-Z scores based on the World Health Organisation (WHO) BMI for age reference values (5–19 years). Based on the BMI Z-score, patients were classified as:

underweight/normal weight with Z-scores between −2 and +0.99, overweight from 1 to 1.99, obese from 2 to 2.99, and very obese ≥ 3**.** (Monasor-Ortolá et al., 2021)

We calculated the change in each participant's BMI Z-score during the treatment period of 12 months through the difference between BMI Z-score at baseline and the last visit. A BMI-Z score increase of 0.5 points at 12 months was considered clinically significant. (Wink et al., 2014)

We defined three groups' patients: patients with an increase in BMI-Z score, patients with a BMI-Z score decreased or patients with a stable BMI-Z score. Fisher–Freeman–Halton exact test, was applied to detect a different distribution of characteristics between three groups.

The association between polymorphisms and BMI-Z score changes was analysed by calculating odds ratios (ORs), 95% confidence intervals (95%CIs), and corresponding P-values. Deviation from Hardy-Weinberg equilibrium for the polymorphisms under investigation was evaluated by comparing expected to observed genotype frequencies using the chi-square test. All P-values were two-sided, and those of less than 0.05 were considered to be statistically significant.

For in vitro experiment, a comparison of multiple groups was performed by one-way ANOVA followed by a post hoc Tukey's test. The results are expressed as means ± SEM of the indicated n values. A P value < 0.05 was considered significant [* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$]. The GraphPad Prism software was used for statistical analysis and graphical representation.

RESULTS

1. Study population

Demographic and Clinical Parameters of the Studied Patients

The recruitment of patients started in January 2019. So far, 209 pediatric patients have been enrolled. The baseline demographic and clinical characteristics are presented in table 1. The study sample consisted of 122 patients, aged 6–17 (median age 13 years, 1st–3rd quartiles 10–16 years). The diagnostic distribution in the patients (67% boys and 32% girls) shows the most reported was autism (n=24; 20%), followed by behavior disorder (n=15; 12%) and attention deficit hyperactivity disorder (ADHD) (n=11; 9%). Patients were treated with antipsychotic monotherapy (risperidone 65 % or aripiprazole 33%).

Sample size, n	**122**
Gender, n (%)	
Male	67
Female	32
Age,years, Me (Q1;Q3)	13 (10;16)
Antipsychotics drug, n (%)	
Risperidone	65
Aripiprazole	33
Diagnosis	
ADHD	11
Intellectual disability	2
Behavior disorder	15
Epilepsy 7q35, dup 2p25.3	1
Bipolar disorder	3
Cyclotymia disorder	1
Anxiety disorder	3
Functional Neurological disorder	1
Behavior Disorder, epilepsy, deletion chromosome 13q	1
Adjustment disorder with mixed disturbance of emotions and conduct	7
Adjustment disorder with anxiety and depressed mood	1
Disruptive mood disorder, ADHD, drug resistant epilepsy	1
Autism spectrum disorder	24
Anxiety and depression disorder	2
Social anxiety disorder	2
Disruptive disorder	5
Obessive compulsive disorder	13
Drug resistant epileptic encephalopathy	1
Drug resistant focal epileptic encephalopathy, aggressive behavior	1
Acute psychiatric episode	1
Psychosis NAS	20
Developmental delay, drug resistant epilepsy	1
Schizofrenia	3
Down Syndrome	1
Tourette	1

Table 1. General characteristics of the paediatric studied at baseline.

BMI-Z changes

Considering the paediatric use of risperidone and aripiprazole and their metabolic adverse effect on weight, one practical way to analyze the weight change during the treatment is to examine the BMI-Z score trend. To this end, we measured the BMI-Z at the study baseline and at the last visit and we calculated the change in each participant. An increase or decrease of 0.5 points at 12 months was considered clinically significant. (Wink et al., 2014).

Among the 122 patients, an increase of BMI-Zscores was observed in 46 (38%) and a decrease in 27 (22%); no variations were observed in 49 (40%) of patients. The relevant characteristics of the three groups are summarized in table 2.

Parameter	Patients with BMI-Z increase	Patients with BMI-Z decrease	Patients with BMI-Z stable
Gender, n (%)			
Male	27 (58,69)	19 (70,37)	36 (73,46)
Female	19 (41,3)	8 (29,62)	13(26,53)
Antipsychotics drug, n (%)			
Risperidone	31 (67,3)	18 (66,6)	31 (63,2)
Aripiprazole	14 (30,4)	8 (29,6)	19 (38,7)
BMI-Z score at baseline	1,01(-0,82; 1,77)	1,08 (0,48;1,98)	1,73 (0,60; 2,60)
BMI-Z score at last visit	1,70 (0,69;2,42)	0,55 (-0,19;1,66) **	1,73 (0,60; 2,60)

*Table 2. Patients and sex are described as numbers with percentages; continuous variables are reported as medians, with first and third quartiles. The frequency of discrete variables was compared across groups by chi-square tests with subgroup comparisons. *Indicate values significantly different from those of the other groups **($p < 0.001$). The distribution of continuous variables was compared across groups by Mann– Whitney tests.*

Since the BMI-Z score increase must be considered clinically relevant, all analyses were performed by comparing the two groups, BMI Z-score decrease and BMI-Z score stable, to the BMI Z- score increase group.

There were no significant differences between the three groups concerning gender distribution (BMI Z-score decrease vs increase $p = 0.45$; BMI Z-score stable vs increase $p = 0.14$) and taken drugs (BMI Z-score decrease vs increase $p = 1.00$; BMI Z-score stable vs increase $p = 0.52$).

For each group, the median BMI-Z scores at the baseline and last visit were reported. The group with reduced BMI Z-score had a significant reduction ($p = 0.0013$ vs the BMI increase group).

Then, according to the Z-score patients were classified as follows: underweight/normal weight: Z-scores between −2 and +0,99, overweight: from 1 to 1,99, obese: from 2 to 2,99, and very obese ≥3.