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.

The percentage of subjects for each category, at baseline and final visit, for each group were shown in figure 1 and table 3.

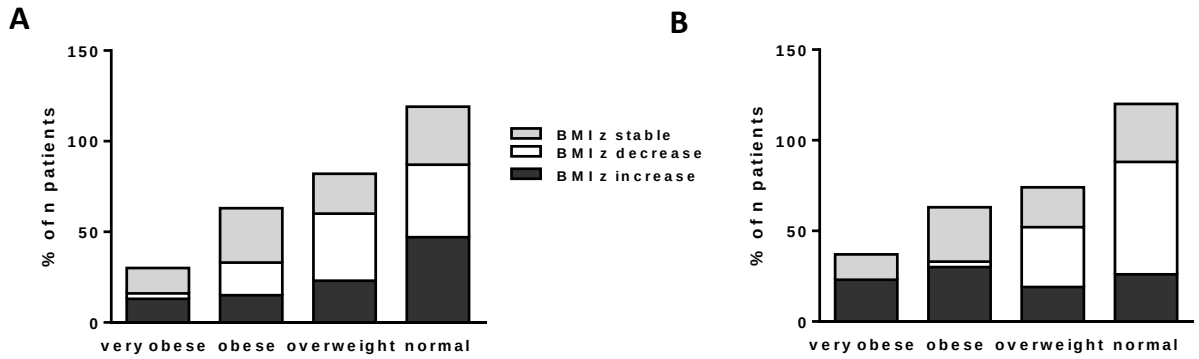

Figure 1. Percentage distribution of patients for each category according to nutritional status classification by BMI Z-score for the three groups. A) at the first visit. B) at the last visit.

Parameter	Patients with BMI-Z increase	Patients with BMI-Z decrease	Patients with BMI-Z stable
Very obese n (%)			
at baseline	6 (13)	1 (3,7)	7 (14,3)
at last visit	11 (23,9)	0 (0)	7 (14,3)
Obese n (%)			
at baseline	7 (15,2)	5 (18,5)	15 (30,6)
at last visit	14 (30,4)	1 (3,7)	15 (30,6)
Overweight n (%)			
at baseline	11 (23,9)	10 (37)	11 (22,4)
at last visit	9 (19,5)	9 (33,3)	11 (22,4)
Normal n (%)			
at baseline	22 (47,8)	11 (40,7)	16 (32,6)
at last visit	12 (26)	17 (62,9)	16 (32,6)

Table 3. Numbers of subjects and percentages for each category according to nutritional status classification by BMI Z-score for the three groups.

In the group with increased BMI-Z score, at the last visit, 18 patients showed an increase in the BMI-Z score such as to produce a change in the class of affiliation. (3/46 from normal to overweight, 4/46 from normal to obese, 1/46 from normal to very obese, 7/46 from overweight to obese, and 3/46 from obese to very obese).

In the group with a decreased BMI-Z score, at the last visit, 10 patients showed a decrease in the BMI-Z score such as to produce a change in the class of affiliation. (1/27 from very obese to obese, 5/27 from obese to overweight, and 4/27 from overweight to normal).

CYP2D6 Genotyping and correlation with BMI

To define whether the changes of BMI-Z were connected with an alteration of drug metabolism which correlates with drug plasma concentration and response in terms of efficacy and/or adverse effects, we performed a pharmacogenetic analysis to detect SNPs in the gene coding for CYP2D6, the most relevant CYP isoform involved in the metabolism of risperidone and aripiprazole.

All CYP2D6 variants analyzed are reported in the Materials & Methods section. The analysis of genotype is important to define the patient's phenotype through the definition of the Activity Score which allows the grouping of individuals in different phenotypic classes based on a score between 0 and 2,5 (Crews et al., 2021). The distribution of phenotypic frequencies and the percentage of subjects for each class was reported in table 4.

Phenotype	AS	Patients with BMI-Z increase	Patients with BMI-Z decrease	Patients with BMI-Z stable
Ultrarapid metabolizer n (%)	> 2,5	3 (7,5)	1 (4,16)	3 (6,6)
Normal metabolizer n (%)	2,25-1,5	31 (77)	18 (74)	29 (64)
Intermediate metabolizer n (%)	0,5-1	6 (15)	5 (20)	13 (29)
Poor metabolizer n (%)	0	0	0	0

Table 4. Distribution and percentage of predicted CYP2D6 phenotypes and activity scores in patients groups. The frequency of phenotype was compared across groups by chi-square tests with subgroup comparisons.

The distribution of phenotypic frequencies for each group shows an extremely high frequency of the normal metabolizer phenotype (77%; 74%; 64 % Figure 4). We did not observe poor metabolizer subjects while individuals with an ultrarapid phenotype are only 7 (7.5 %, 4.16 %, 6.6 % for each group, respectively). The distribution of CYP2D6 metabolizer phenotypes in the three subgroups studied showed no significant differences (BMI decrease, Fisher's exact test $p = 0.4038$; BMI-Z stable, Fisher's exact test $p = 0.6432$ vs BMI increase group).

Analysis of SNPs related to SGA pharmacodynamic and correlation with BMI

These data suggest that the interpersonal variability in terms of BMI change observed after SGA administration depend on the presence of polymorphisms in selected candidates' genes, coding for receptors drug targets, that influence the pharmacodynamics of SGAs and are highly associated with food intake, metabolism, and body weight variations (Zhang et al., 2016).

All SNPs were analyzed by qPCR and the differences in genotype frequencies were reported in table 5. The genotype frequencies are statistically in equilibrium with the Hardy-Weinberg law (p> 0,05)

except for the HTR2C gene because of its location on the X chromosome. Thus, for this gene, the prevalence of genotypes of the polymorphic variants was analyzed separately in males and female.

Gene	Variant	Genotype	Patients	p value > 0,05
ADRA2A	rs1800544 (c.-1252G>C)	GG GC CC	6 (0,05) 38 (0,37) 58 (0,56)	0,998
ADRB3	rs4994 (c.190T>C)	TT TC CC	90 (0,88) 12 (0,11) 0 (0)	0,895
DRD2	rs1799732 (c.-486_-485insC)	-/- -/C CC	0 (0) 14 (0,13) 88 (0,86)	0,329
DRD2	rs6275 (c.939T>C)	TT TC CC	16 (0,16) 43 (0,44) 37 (0,38)	0,845
DRD2	rs7131056 (c.-32+16024T>G)	TT TG GG	16 (0,16) 41 (0,40) 45 (0,44)	0,449
DRD2	rs1799978 (c.-585A>G)	AA AG GG	86 (0,84) 16 (0,16) 0 (0)	0,691
HTR2A	rs6313 (c.102C>T)	CC CT TT	31 (0,30) 50 (0,50) 21 (0,20)	0,995
HTR2C	rs3813929 (c.-759C>T)	CC CT TT	75 (0,73) 6 (0,05) 21 (0,20)	3,16E-16
HTR2C	rs6318 (c.68G>C)	GG GC CC	71 (0,69) 13 (0,07) 18 (0,16)	4,15E-10
HTR2C	rs518147 (c.-697C>G)	CC CG GG	47 (0,46) 11 (0,10) 44 (0,43)	2,41E-14
HTR2C	rs1414334 (c.551-3008C>G)	CC CG GG	11 (0,14) 15 (0,14) 76 (0,74)	2,27E-06
MCR4	rs17782313 (g.60183864T>C)	TT TC CC	57 (0,55) 35 (0,34) 9 (0,08)	0,575
MCR4	rs489693 (g.60215554C>A)	CC CA AA	48 (0,47) 42 (0,41) 12 (0,11)	0,836
DRD3	rs6280 (c.25G>A)	GG GA AA	7 (0,06) 46 (0,45) 49 (0,48)	0,685
HTR6	rs1805054 (c.267C>T)	CC CT TT	68 (0,66) 31 (0,30) 3 (0,02)	0,972

Table 5. Genotype frequencies and Hardy-Weinberg equilibrium test of selected SNPs in the study population.

Results of associative analysis of genotypes frequency and changes in BMI-Z score (increase or decrease) were carried out using the odds ratio (OR) method. This will allow us to evaluate if the presence of specific variant alleles increases or decreases the risk of weight gain. .

Between the 15 SNPs studied, the variants statistically significant associated with BMI changes were shown in table 6, for HTR2C males and females were analyzed separately (table 7).

Gene	Variant	Genotype	Odds ratio (95 % CI)	p value
ADRA2A	rs1800544 (c.-1252G>C)	GG	1,00	Ref
		GC	0,8266 (0,4856-1,407)	P = 0,6664
		CC	1,3165 (0,3771-4,5960)	P = 0,4829
		GC+CC	0,8683 (0,5183-1,4546)	P = 0,5916
ADRB3	rs4994 (c.190T>C)	CC	1,00	Ref
		TC	0,9774 (0,0192-49,815)	P = 0,9909
		TT	0,6515 (0,2961-1,4335)	P = 0,2869
		TC+CC	0,6515 (0,2961-1,4335)	P = 0,2869
DRD2	rs1799732 (c.-486_-485insC)	CC	1,00	Ref
		-/C	2,6878 (1,1638-6,2074)	P = 0,0206*
		-/-	1,1509 (0,0226-58,6791)	P = 0,9441
		-/- + -/ C	2,6878 (1,1638 to 6,2074)	P = 0,0206*
DRD2	rs6275 (c.939T>C)	CC	1,00	Ref
		TC	0,2516 (2,0950-7,5422)	P < 0,0001*
		TT	0,4717 (0,1997-1,1139)	P = 0,0865*
		TC+TT	0,2956 (0,1623-0,5382)	P = 0,0001*
DRD2	rs7131056 (c.-32+16024T>G)	GG	1,00	Ref
		TG	1,9626 (1,0795-3,5684)	P = 0,0271*
		TT	7,3286 (2,2921-23,431)	P = 0,0008*
		TG+TT	2,4618 (1,3914-4,3558)	P = 0,0020*
DRD2	rs1799978 (c.-585A>G)	GG	1,00	Ref
		AG	0,9435 (0,0185 to 48,097)	P = 0,9769
		AA	0,6658 (0,2999 to 1,4781)	P = 0,3174
		AG+AA	0,6658 (0,2999 to 1,4781)	P = 0,3174
HTR2A	rs6313 (c.102C>T)	CC	1,00	Ref
		CT	0,8772 (0,4520-1,7025)	P = 0,6985
		TT	1,4118 (0,6183- 3,2233)	P = 0,4130
		CT+TT	1 (0,5316-1,8812)	P = 1,0000
MCR4	rs17782313 (g.60183864T>C)	TT	1,00	Ref
		TC	2,0300 (0,6673-6,2228)	P = 0,2114
		CC	0,5403 (0,3022-0,9659)	P = 0,0378
		TC+CC	0,6651 (0,3833-1,1539)	P = 0,1469
MCR4	rs489693 (g.60215554C>A)	CC	1,00	Ref
		CA	1,1538 (0,3772-3,5296)	P = 0,8019
		AA	5,6842 (1,7748-18,205)	P = 0,0034*
		CA+CC	1,5061 (0,8577-2,6446)	P = 0,1540
DRD3	rs6280 (c.25G>A)	AA	1,00	Ref
		AG	1 (0,5662-1,7662)	P = 1,0000
		GG	1,5319 (0,4061-5,7782)	P = 0,5289
		AG+GG	1,0409 (0,5975-1,8134)	P = 0,8874
HTR6	rs1805054 (c.267C>T)	CC	1,00	Ref
		CT	0,600 (0,1981-1,8174)	P = 0,3663
		TT	0,1022 (0,0045-2,3113)	P = 0,1517
		CT+TT	0,3365 (0,1451- 0,7804)	P = 0,0112

Table 6. Associative Analysis of Genotypes frequency and Body Mass Index in Patients with increase and decrease of BMI-Z score

A

Gene	Variant	Genotype	Odds ratio (95 % CI)	p value
HTR2C	rs3813929 (c.-759C>T)	CC	1,00	Ref
		TT	1,3772 (0.0187 to 48.630)	P = 0.3733
HTR2C	rs6318 (c.68G>C)	GG	1,00	Ref
		CC	0.4402 (0.2149 to 0.9016)	P = 0.0250*
HTR2C	rs518147 (c.-697C>G)	CC	1,00	Ref
		GG	0,7256 (0,4160-1,2655)	P = 0.2583
HTR2C	rs1414334 (c.551-3008C>G)	CC	1,00	Ref
		GG	0,7416 (0.3464-1.5876)	P = 0.4414

B

Gene	Variant	Genotype	Odds ratio (95 % CI)	p value
HTR2C	rs3813929 (c.-759C>T)	CC	1,00	Ref
		CT	0,3434 (0,1730 to 0,6819)	P = 0,0023*
		TT	0,3137 (0,1259 to 0,7820)	P = 0,0129*
		CT+TT	0,3333 (0,1832 to 0,6066)	P = 0,0003*
HTR2C	rs6318 (c.68G>C)	GG	1,00	Ref
		GC	2,300 (1,2329-4,2747)	P = 0,0088*
		CC	1,5152 (0,6786-3,3830)	P = 0,3106
		GC+CC	2,0303 (1,1457-3,5980)	P = 0,0153*
HTR2C	rs518147 (c.-697C>G)	CC	1	Ref
		CG	5,7093 (2,5571-12,747)	P < 0,0001*
		GG	4,4563 (2,2034-9,0127)	P < 0,0001*
		CG+GG	4,8824 (2,5421-9,3770)	P < 0,0001*
HTR2C	rs1414334 (c.551-3008C>G)	CC	1,00	Ref
		CG	1,381 (0,7904-2,4128)	P = 0,2569
		GG	1,188 (0,0231-61,1692)	P = 0,9316
		CG+GG	1,381 (0,7904 to 2,4128)	P = 0,2569

Table 7. Associative Analysis of HTR2C SNPs Genotypes frequency and Body Mass Index in Patients with increase and decrease of BMI-Z score. (A)Male patients;(B)Female patients

The percentage of patients with BMI-Z score increase or decrease according to the three genotypes for SNPs significantly associated with the change is shown in figure 2 and 3.

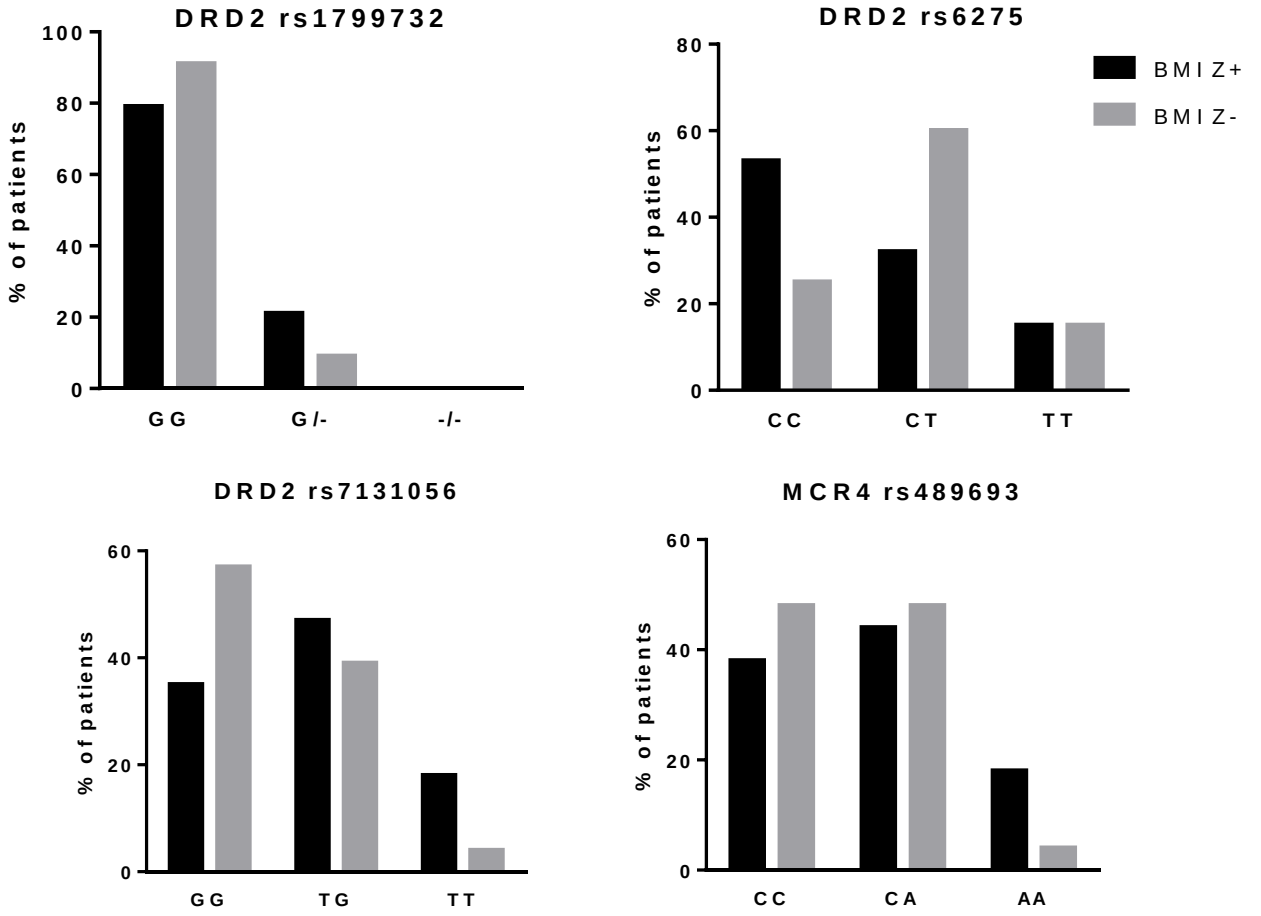

Figure 2. Graphical representation of percentage distribution of genotype for polymorphisms significantly associated with BMI-Z score change in two groups BMI-Z score increase and BMI-Z score decrease.

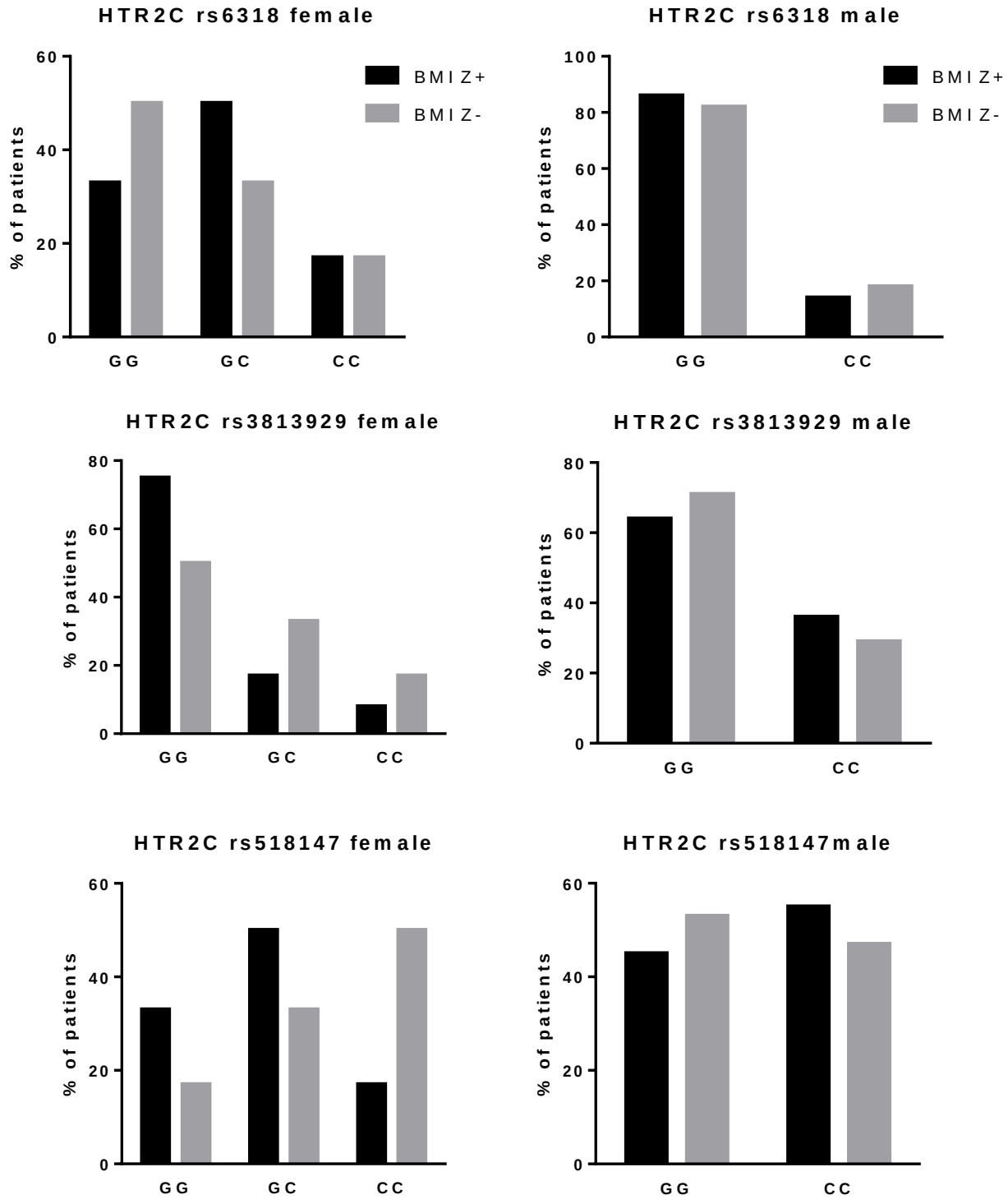

Figure 3. Graphical representation of percentage distribution of genotype for HTR2C polymorphism

2. In vitro study

SW872 cell line as a model of adipogenic differentiation: morphological changes and gene expression profile

The process of differentiating SW872 cell lines in vitro requires a pharmacological cocktail containing glucocorticoids (dexamethasone), cAMP stimulating agent (IBMX − 3-Isobutyl-1-methylxanthine), and insulin to induce both inhibitions of growth and induction of the expression of transcriptional factors controlling adipogenesis (Fiorani et al., 2021). To evaluate the course of adipogenesis first we assessed changes in lipid content by comparing differentiated cells (exposed to the cocktail) and undifferentiated cells (exposed to the vehicle) at 3 - 6 - 10 days of incubation with confluent preadipocytes (T0). The staining with Oil red O, a fat-soluble dye that stains neutral triglycerides and lipids, revealed a significant increase in the lipid content at 6 - 10 days compared to T0 (Figure 4).

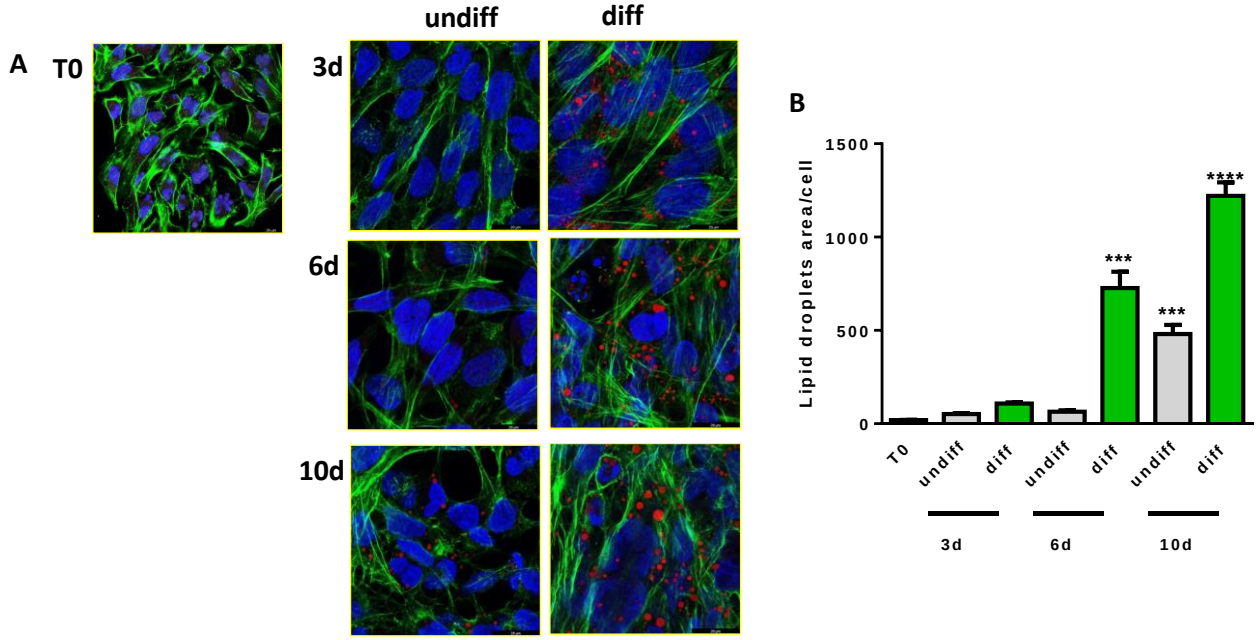

*Figure 4. Confocal microscopy images of oil red immunofluorescent staining of undifferentiated (undiff) and differentiated (diff) SW872 cell line at defined time point. Scale bar = 20 µm. DAPI (blue) and phalloidin (green) were used for nuclei and cytoskeleton detection, respectively (B) Lipid droplet content (area fraction stained) is shown in the graph. Values are expressed as mean ± standard error. *** $p < 0.001$; **** $p < 0.0001$ vs T0*

To confirm adipogenic differentiation we analyzed the expression of key adipogenic markers, *i.e.* CEBP beta, CEBP delta, CEBP alpha, and PPAR gamma of undifferentiated and differentiated and

preadipocytes at 3 - 6 - 10 days of culture. We observed that the expression of the differentiation marker began to increase on the third day of differentiation with a significant increase, especially on day 6, and remain at a similar level on day 10 compared to day 0. (Figure 5)

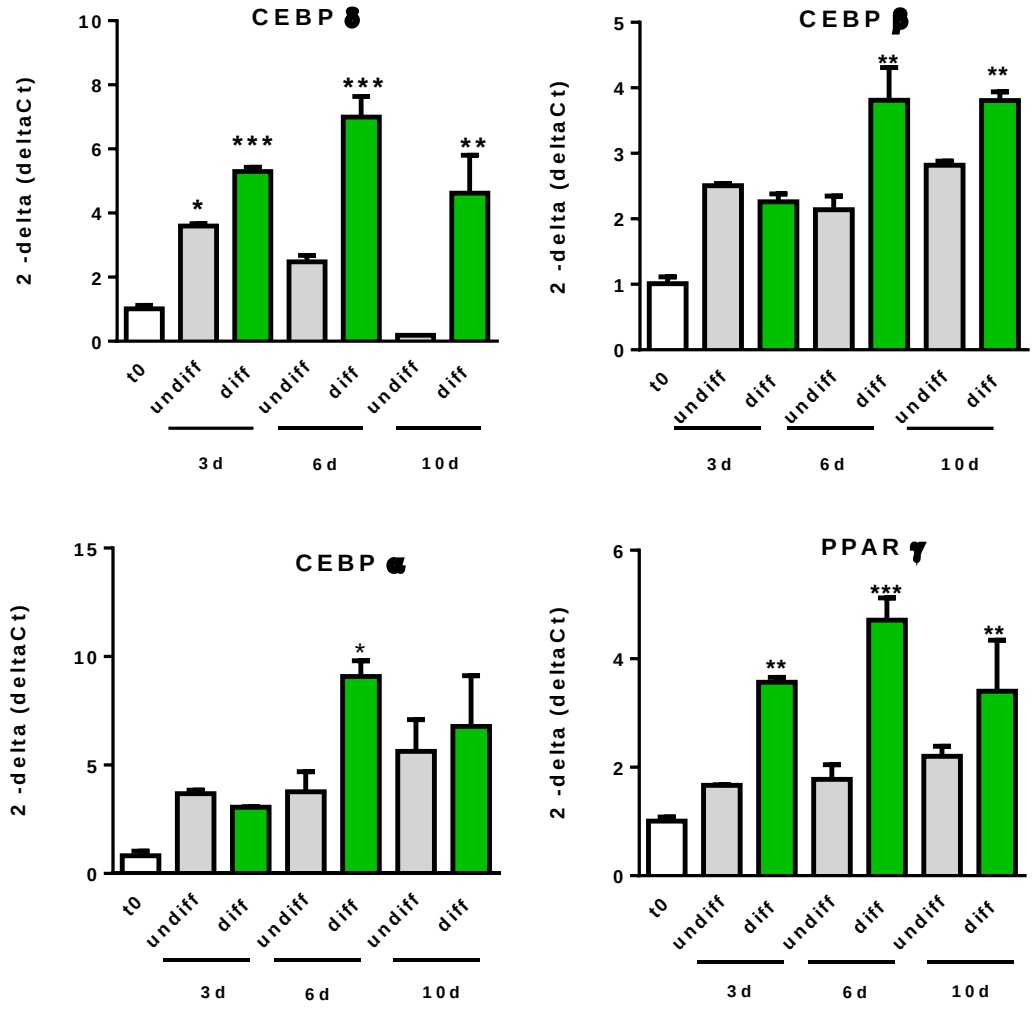

*Figure 5. Effect of the differentiation cocktail on adipogenic gene expression. Relative mRNA expression of nuclear-encoded genes CEBP beta, CEBP delta, CEBP alpha, and PPAR gamma.The expressionof each gene was normalized to the expression of reference genes GAPDH and RPL32. Values are expressed as mean ± SEM (n ≥ 3 for each group). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. T0*

Cell viability of SW872 in the presence of Risperidone

To clarify the mechanism of risperidone-induced weight gain, we decided to investigate its possible effect on the adipogenic differentiation process in our cell model. First, to define the concentration of the drug to be used in the differentiation experiment we examined the effect of increasing concentration of risperidone on cell viability of SW872.

As shown in Figure 6, 1µM risperidone did not affect cell viability of SW872 preadipocytes while 10 µM risperidone induced a significant decrease; therefore, 1µM of risperidone was used in the following in vitro experiments.

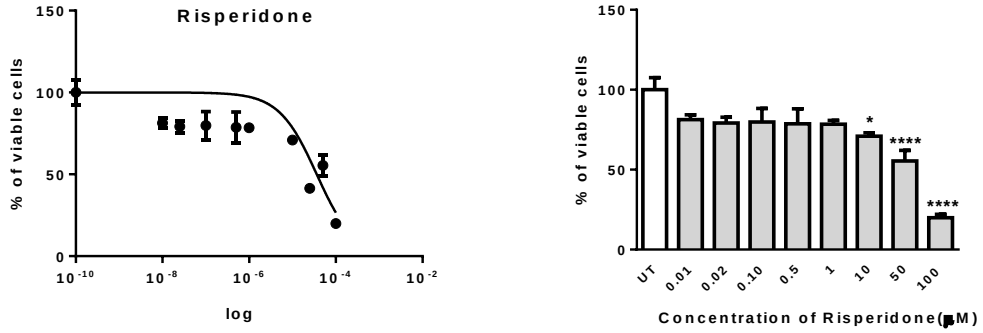

*Figure 6. Risperidone cytotoxicity evaluation in SW872 undifferentiated cells. Cell viability at 48h was measured by MTT assay. * p < 0,05; **** p < 0,0001. vs time 0*

Risperidone promotes the differentiation of preadipocyte

To understand whether risperidone could have an effect on adipocyte differentiation and lipid accumulation, we evaluated changes in lipid content and expression of key adipocyte markers at 3 - 6 - 10 days of incubation. To this end, cells were treated with risperidone every 24 hours (risp in the following figures). As a positive control of differentiation, we used the cells treated with the differentiation cocktail (diff in the following figures) and as negative control cells treated with the vehicle (undiff in the following figures).

After Oil red O staining, we observed that risperidone alone was able to promote lipid accumulation with a significant increase, especially on day 6 compared to undifferentiated cells. (figure 7)

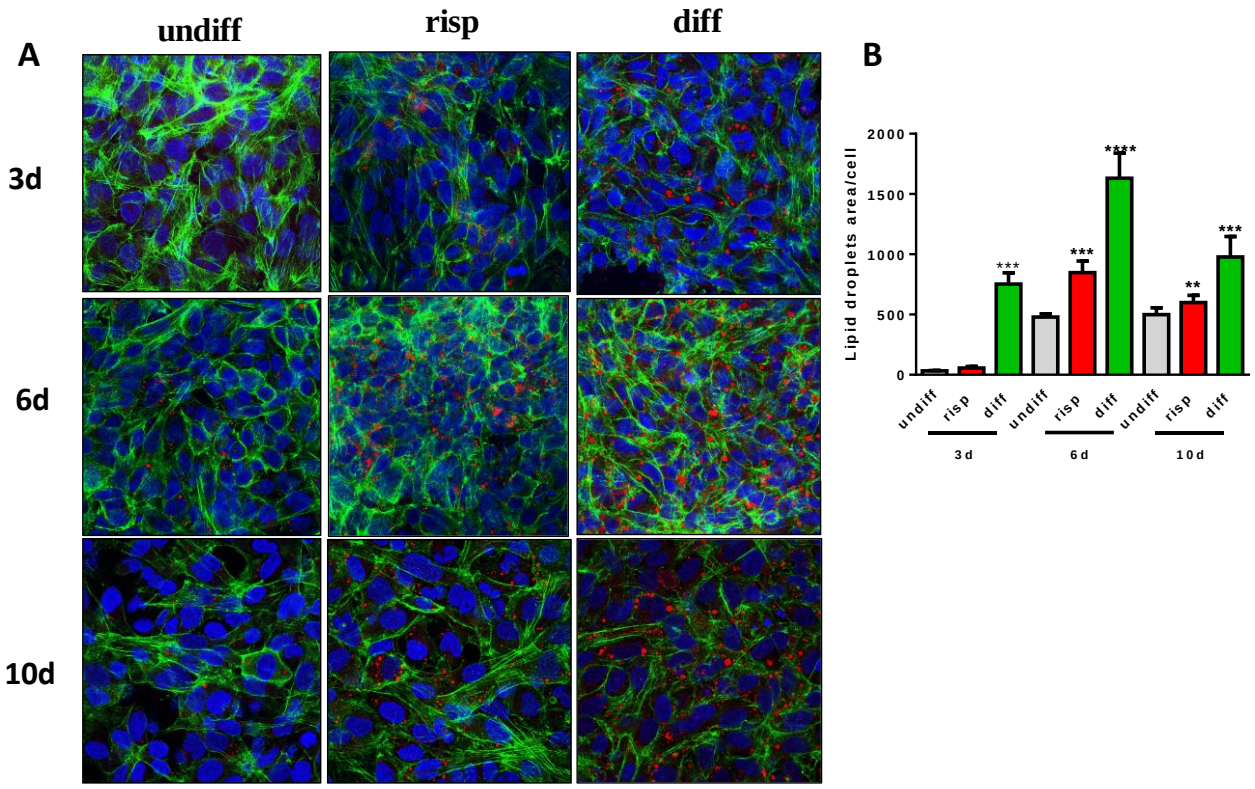

Figure 7. *Confocal microscopy images of oil red immunofluorescent staining of vehicle-treated (undiff); risperidone treated (risp) and differentiated (diff) SW872 cell line at defined time point. Scale bar = 20 µm. DAPI (blue) and phalloidin (green) were used for nuclei and cytoskeleton detection, respectively (B) Lipid droplet content (area fraction stained) is shown in the graph. Values are expressed as mean ± standard error. * p < 0.05; ** p < 0.01; *** p < 0.001 vs. vs T0*

This data was confirmed by the analysis of the transcript levels of adipogenic differentiation genes. We found that risperidone promoted a significant increase of all adipogenic biomarkers significantly at day 6 of differentiation (Figure 8).

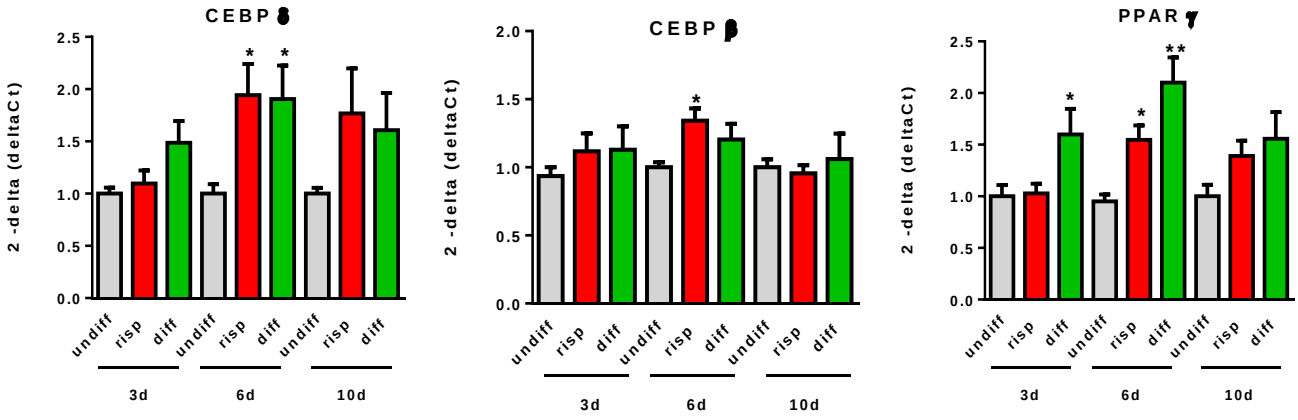

Figure 8. Effect of risperidone on adipogenic gene expression. Relative mRNA expression of nuclear-encoded genes CEBP beta, CEBP delta and PPAR gamma.The expression of each gene was

*normalized to the expression of reference genes GAPDH and RPL32. Values are expressed as mean ± SEM (n ≥ 3 for each group). * p < 0.05, ** p < 0.01, vs. T0.*

To better understand the phenotype of risperidone-induced adipocytes, next, we assessed the molecular expression levels of known brown adipocyte markers, PRMD16 and UCP1. The downregulation of these genes seems to be associated with a higher risk of type 2 diabetes mellitus (T2DM) (Mishra et al., 2021). Compared to classically differentiated cells risperidone-induced adipocytes showed a significantly lower expression of both biomarkers (Figure 9). These results suggested that risperidone alone might have a role in the definition of the phenotype of adipocytes.

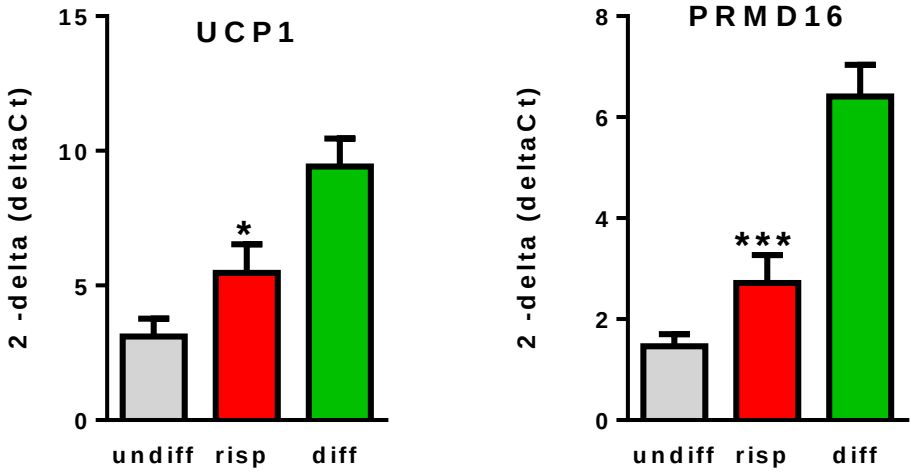

*Figure 9. Effect of risperidone treatment on brown adipocyte markers gene expression. Relative mRNA expression of UCP1 and PRMD16 in vitro differentiated and risperidone-treated SW872 adipocytes at 6 days. The expression of each gene was normalized to the expression of reference genes GAPDH and RPL32. Values are expressed as mean ± SEM (n ≥ 3 for each group). * p < 0.05, *** p < 0.001 vs. T0.*

Risperidone effect on Mitochondrial Bioenergetic Profile

PRDM16 regulates UCP1 expression in brown adipocytes. Brown and beige adipocytes are characterized by a high density of mitochondria that contain uncoupling protein 1 (UCP1) in their inner membrane. UCP1 permits proton leak across the inner mitochondrial membrane (Klingenberg et al, 1999). Dissipation of the mitochondrial proton gradient by UCP1 drives the oxidation of fatty acids to dissipate and produce energy as heat, a phenomenon called “non-shivering adaptive thermogenesis, which is the signature feature of brown adipocytes.

Since mitochondrial respiration and uncoupling are hallmarks of brown adipocytes, we measured mitochondrial bioenergetic profiles to better understand the phenotype of risperidone-induced cells.

Risperidone adipocytes exhibited a decreased level of OxPhos ATP and ATP-linked respiration (L-R) (Figure 10A). No modifications were detected in the production of glycolytic ATP between the two groups (Figure 10B). Moreover, basal respiratory capacity as well as maximal respiration showed a trend toward reduction in risperidone-treated cells (Figure 10B).

Taken together, these data indicate that risperidone altered the bioenergetic profile of the cells. It especially decreases the capability of the cells to produce energy by impairing the proper coupling between the respiratory chain and ATP production, therefore conferring a profile more similar to white adipocytes in terms of reduced basal respiration and coupling activity.

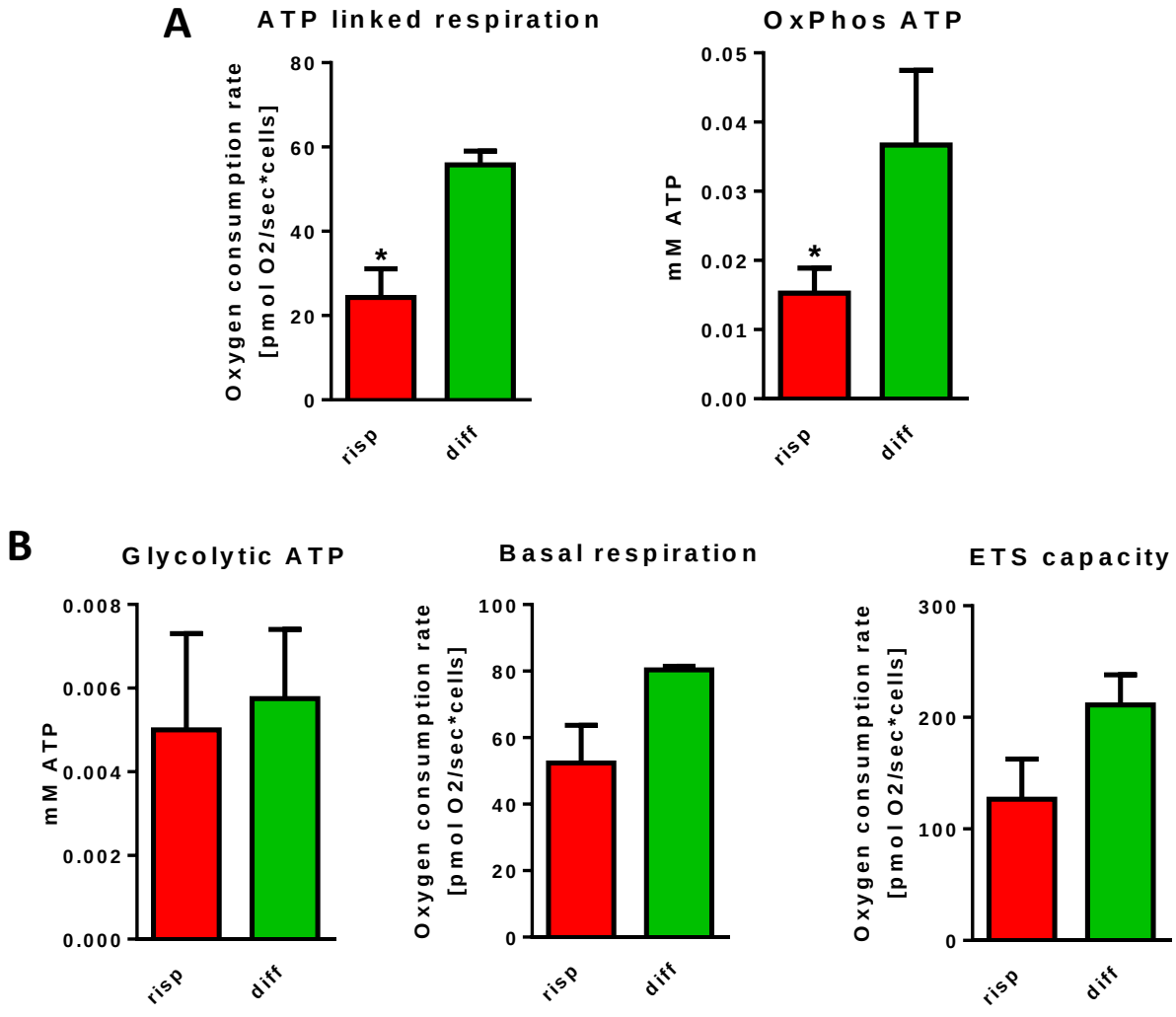

Figure 10. Effect of risperidone on mitochondrial respiration. (A) ATP production (Basal respiration-Leak respiration copulated to ATP production), and measurement of ATP production through

oxidative phosphorylation by mitochondria (at 10 min after substrate addition and normalized on the value at time 0) in vitro differentiated and risperidone-treated SW872 adipocytes at 6 days. $p < 0.05$; (n=4).(B) Measurement of ATP production through glycolysis in vitro differentiated and risperidone-treated SW872 adipocytes at 6 days. Values are expressed as the ATP produced at 10 min after substrate addition and normalized on the value of ATP at time 0. Basal respiratory capacity and maximal respiration, in vitro, differentiated, and risperidone-treated SW872 adipocytes at 6 days; * $p < 0.05$; (n=4).*

Risperidone effect on Mitochondrial Biogenesis

Since the increased abundance of mitochondria is a hallmark of browning, we next analyzed the levels of one of the key mitochondrial biogenesis regulators, PGC-1alpha. The expression level of PGC-1alpha was significantly reduced with risperidone compared to differentiated cells (Figure 11A).

Several studies have provided evidence that in the white adipose tissue of obese patients, PGC-1α expression is attenuated, suggesting that the downregulation of PGC-1α is associated with an obesity-related disturbance (Kobayashi et al., 2021).

Accordingly, we assessed mRNA levels of a key marker of white adipocytes such as Hox4 (Figure 11B). Interestingly, Hox4 displayed a significantly increased expression level on risperidone-treated cells compared to the classically differentiated ones (Figure 11B).

Taken together these results suggest that risperidone could induce differentiation and direct adipocyte toward an intermediate profile more similar to the white one.

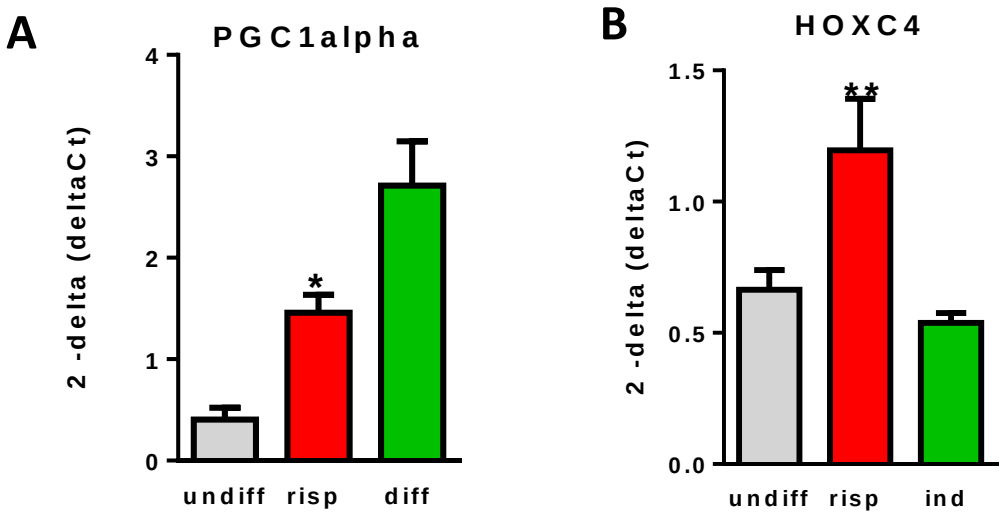

*Figure 11. Risperidone effect on Mitochondrial Biogenesis. (A)(B) Relative mRNA expression of PGC1alpha and HOX 9 in vitro differentiated and risperidone-treated sw872 adipocytes at 6 days.(B) Relative mRNA expression of UCP1 and PRMD16 in vitro differentiated and risperidone-treated sw872 adipocytes at 6 days. Gene expression results were normalized to the expression of reference genes GAPDH and RPL32. Values are expressed as mean ± SEM (n ≥ 3 for each group). * $p < 0.05$, ** $p < 0.01$, vs. T0.*

SNP generation in SW872 cells: A model for the study of SNP role in SGAs-induced adipogenesis

To clarify the role of the selected SNPs identified in our population study as related to BMI changes after SGAs administration, we generated cell clones expressing genes carrying the SNPs. To this end, we used the site-directed mutagenesis approach to modified the genes to be transfected in SW872 (see material and methods section). (H. Han et al., 2020). We selected two SNPs to be inserted in the cells: rs6318 for the 5-HTR2C gene and rs6275 for the DRD2 gene. These SNP were already studied in correlation with weight gain, metabolic ADRs, and antipsychotic treatment with a focus on SNC but not at a peripheral level. In addition, we identified a correlation of these SNPs with BMI changes in our population. In order to understand the genotype related to 5-HTR2C and DRD2 genes of SW872, first, we analyzed them by qPCR finding the following genotype: rs6318: GG; rs6275: GG, which are the most common genetic variants for the caucasian population.

Generation and characterization of the trasfected clones

The two variant alleles for each gene were subcloned into a neomycin-resistant pcDNA3.1(+) _myc tag - vector and transfected in SW872. Stably transfected cells were selected in medium supplemented with neomycin and the protein expression of the receptors were visualized by confocal microscopy after the staining with an anti-Myc antibody.(Figure 12-13)

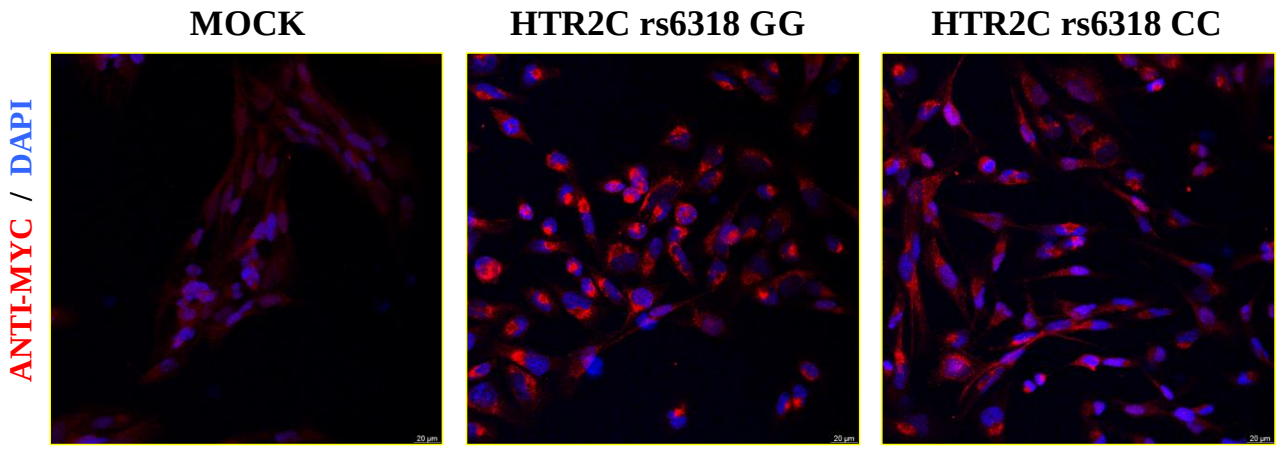

Figure 12. Representative fluorescence images of mock-transfected (empty control vector) and HTR2C GG and HTR2C CC stably transfected clones. Anti-Myc tag staining (red). DAPI (blue) was used for nuclei. Scale bar = 20 µm.

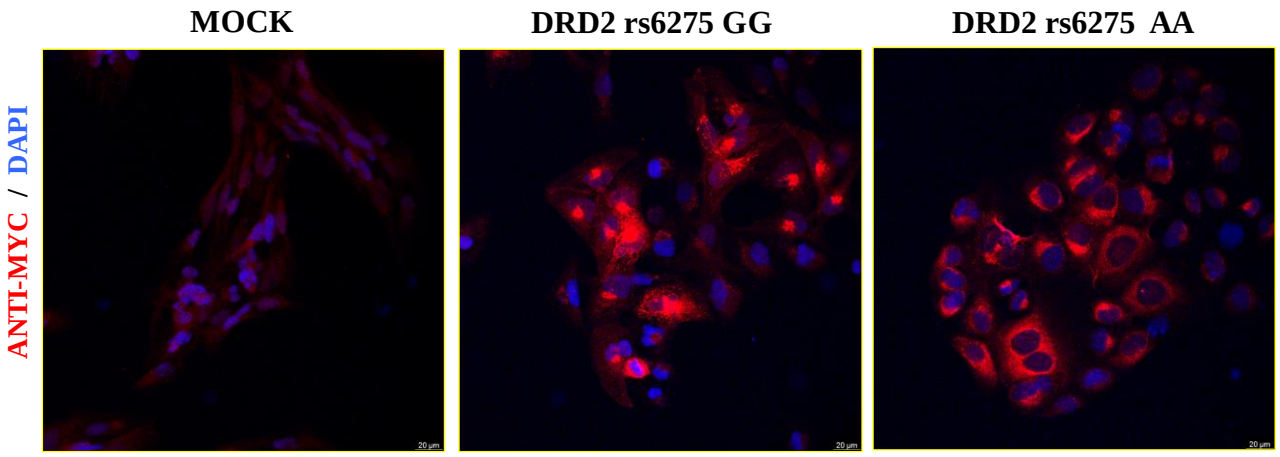

Figure 13. Representative fluorescence images of mock-transfected (empty control vector) and and DRD2 GG and DRD2 AA stably transfected clones . Anti-Myc tag staining (red). DAPI (blue) was used for nuclei. Scale bar = 20 µm.

To confirm the stable transfection, expression levels of the transcript and the protein were analyzed. qPCR and western blotting analysis reveals that the expression of HTR2C (figure 14) and DRD2 (figure 15) were increased in stable clones compared to mock-transfected cells for both SNPs.

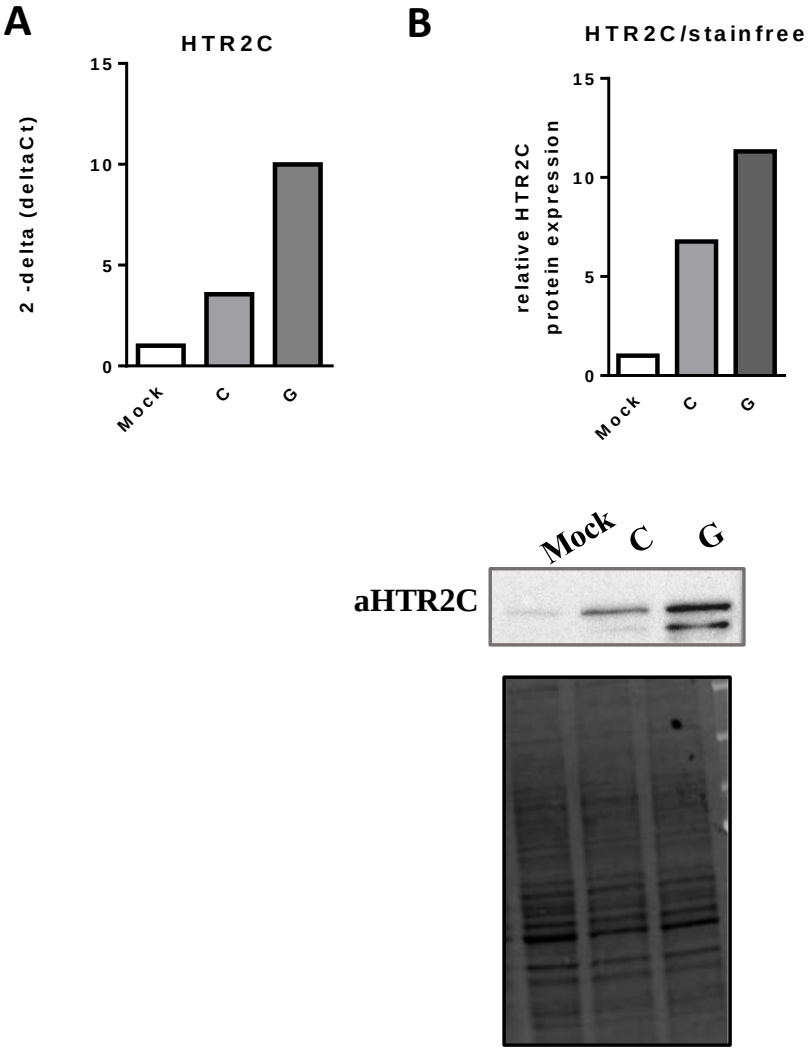

Figure 14. (A) Relative mRNA expression of HTR2C from the stable clone. Data are expressed as fold change over mock-empty clone. (B): Western blot of HTR2C expression in stable clones, with relative quantification, normalized on total proteins

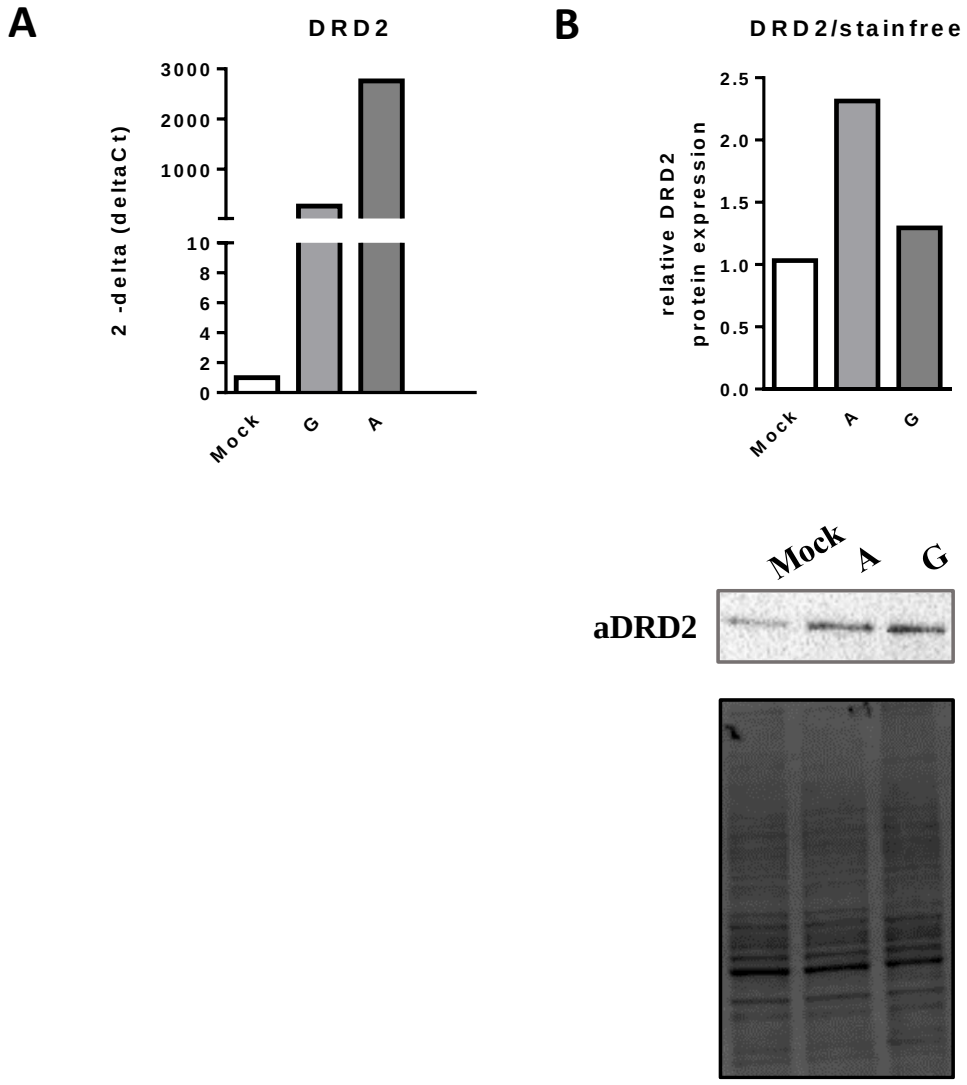

Figure 15. (A)Relative mRNA expression of DRD2 from the stable clone. Data are expressed as fold change over mock-empty clone. (B): Western blot of DRD2 expression in stable clones, with relative quantification, normalized on total proteins

Differentiation of SW872 HTR2C GG and CC stable clones

To check the quality of this model in terms of adipocytic differentiation, we treated the clones with the classical cocktail of differentiation. First, we analyzed the effect of the cocktail on the HTR2C GG and CC clones, in the same condition used for the parental cell line.

After Oil red O staining, we observed a significant increase in the lipid content at 6 - 10 days compared to T0 for both gene variants clones. (Figure 16-17)

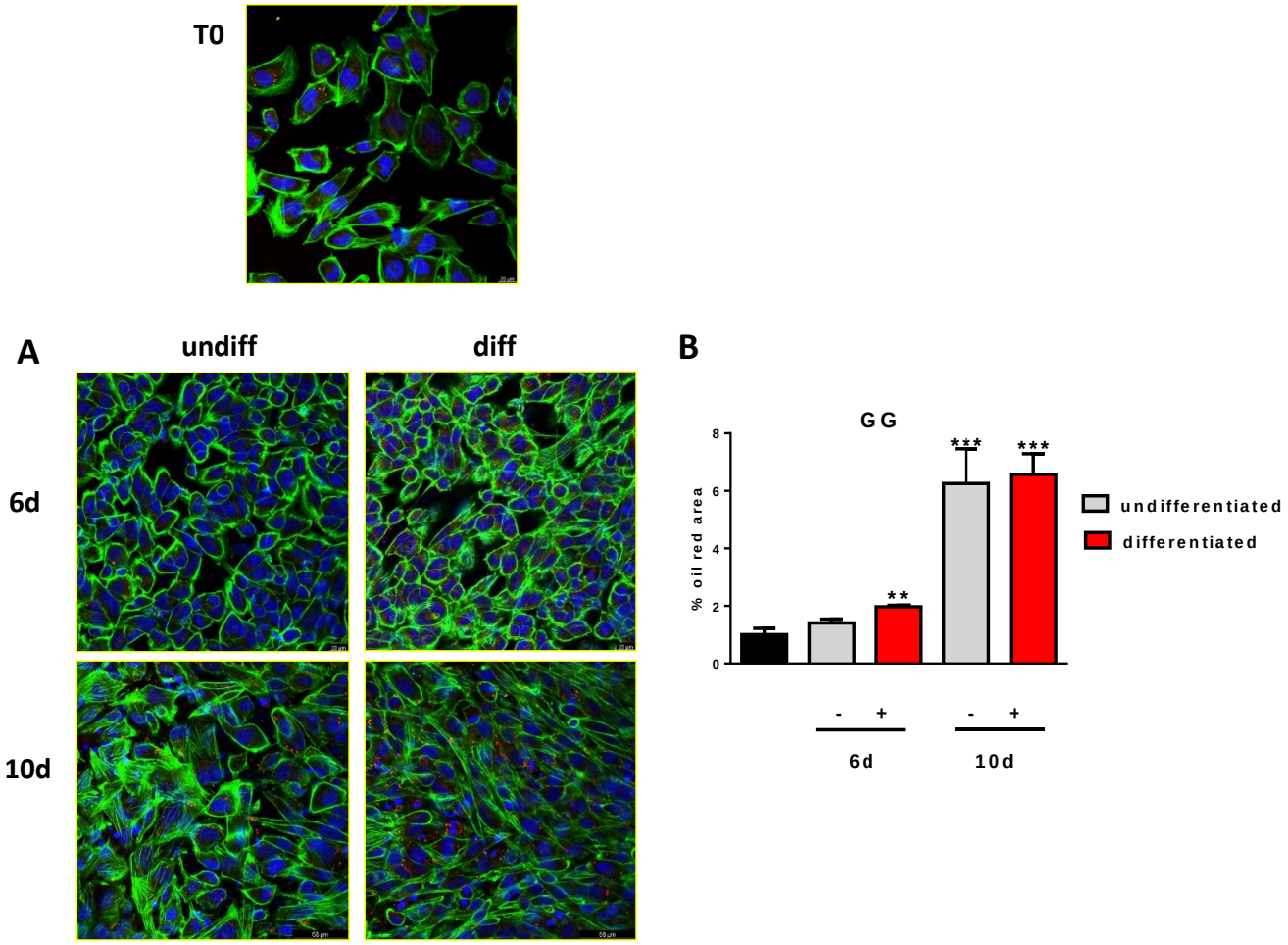

*Figure 16. Confocal microscopy images of oil red immunofluorescent staining of undifferentiated (undiff); and differentiated (diff) sw872 HTR2C GG stable clones at defined time points. Scale bar = 20 µm. DAPI (blue) and phalloidin (green) were used for nuclei and cytoskeleton detection, respectively (B) Lipid droplet content (area fraction stained) is shown in the graph. Values are expressed as mean ± standard error. ** $p < 0.01$; *** $p < 0.001$ vs. vs time 0 for each variant.*

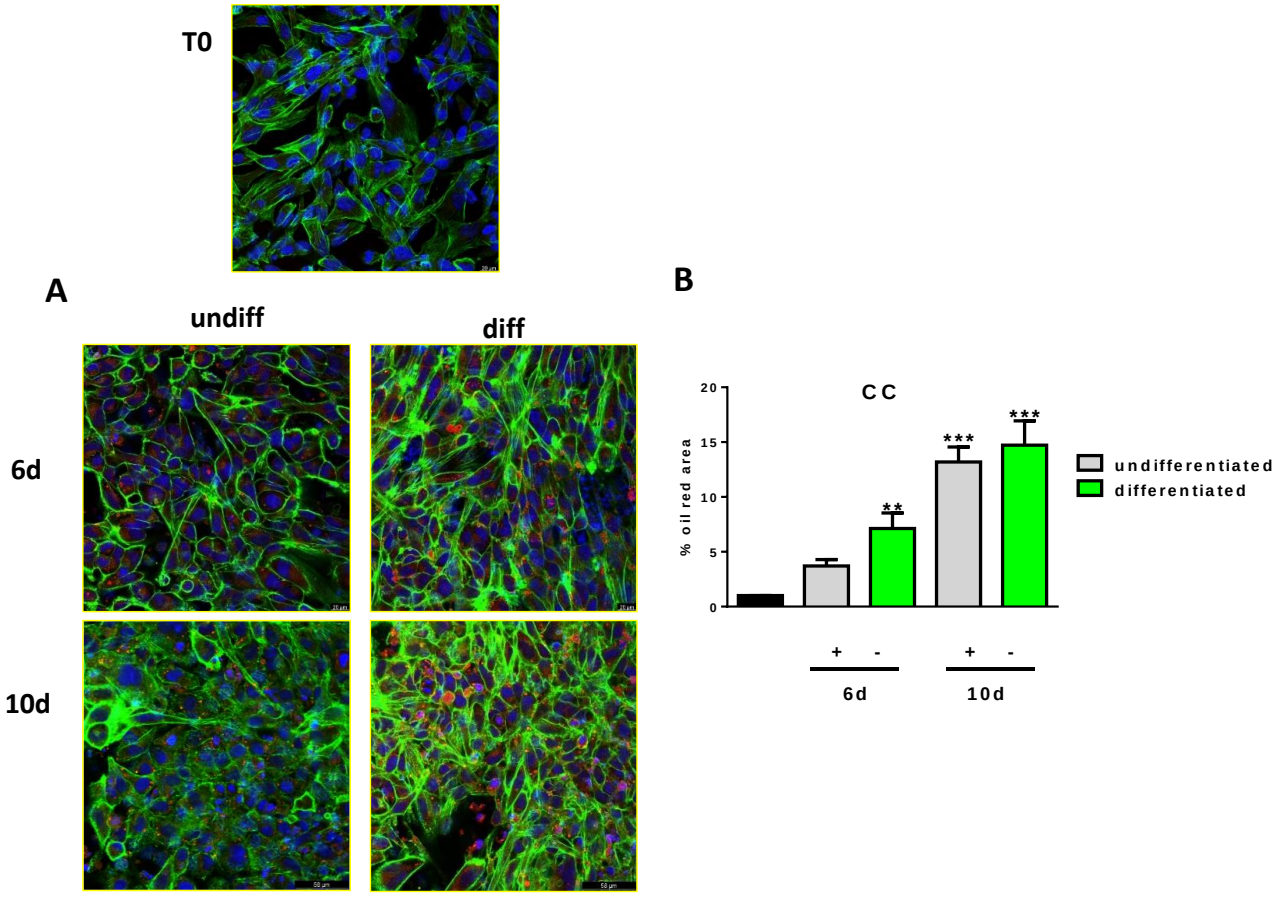

*Figure 17. Confocal microscopy images of oil red immunofluorescent staining of undifferentiated (undiff); and differentiated (diff) sw872 HTR2C CC stable clones at defined time points. Scale bar = 20 µm. DAPI (blue) and phalloidin (green) were used for nuclei and cytoskeleton detection, respectively (B) Lipid droplet content (area fraction stained) is shown in the graph. Values are expressed as mean ± standard error. ** $p < 0.01$; *** $p < 0.001$ vs. vs time 0 for each variant.*

To confirm adipogenic differentiation on a gene expression level, we analyzed the expression of the key adipogenic markers. For HTR2C GG clones, the expression of CEBP beta and CEBP delta in differentiated cells began to significantly increase on the third day of differentiation and remain at a similar level at day 6- 10 days compared to day 0. PPAR gamma and CEBP alfa are both significantly higher in differentiated cells at 6 days. (Figure 18). For HTR2C CC the trend was analogous (figure 19)

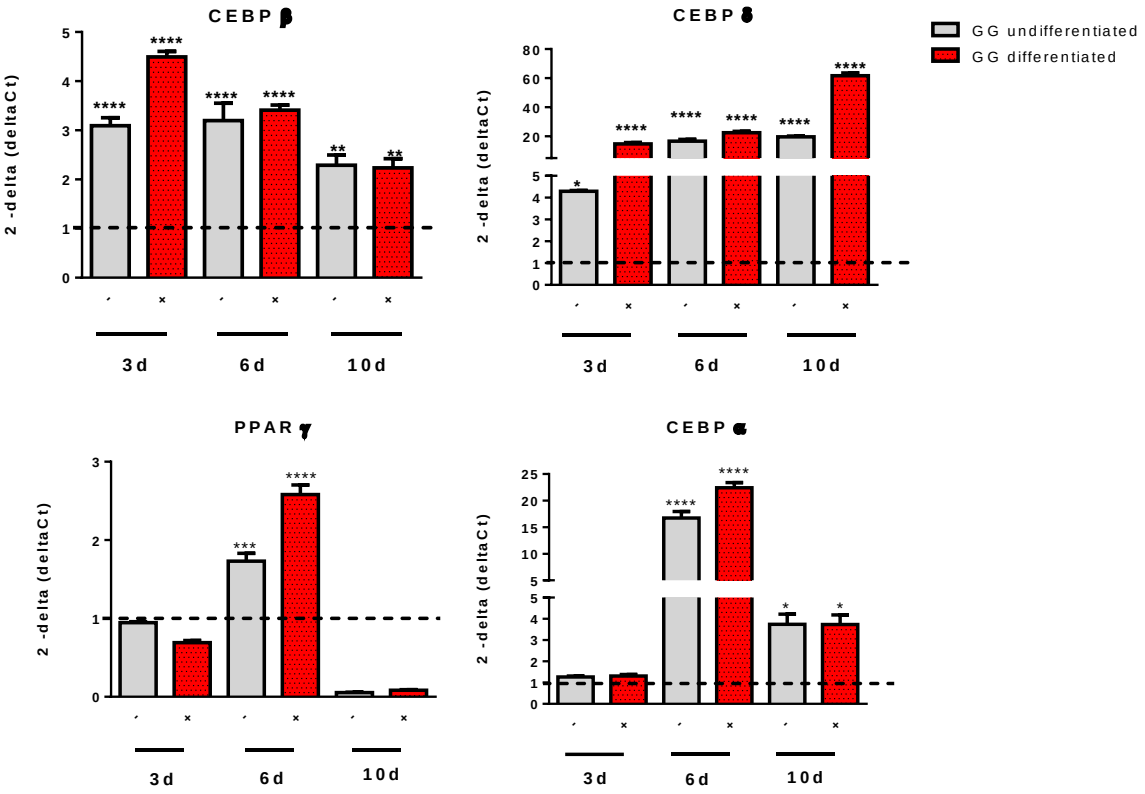

*Figure 18. Effect of differentiation protocols on adipogenesis gene expression. Relative mRNA expression of nuclear-encoded genes CEBP beta, CEBP delta, CEBP alpha, and PPAR gamma in sw872 HTR2C GG stable clone. The expression of each gene was normalized to the expression of reference genes GAPDH and RPL32. Values are expressed as mean ± SEM (n ≥ 3 for each group). * p < 0.05, ** p < 0.01, **** p < 0.0001 vs. T0*

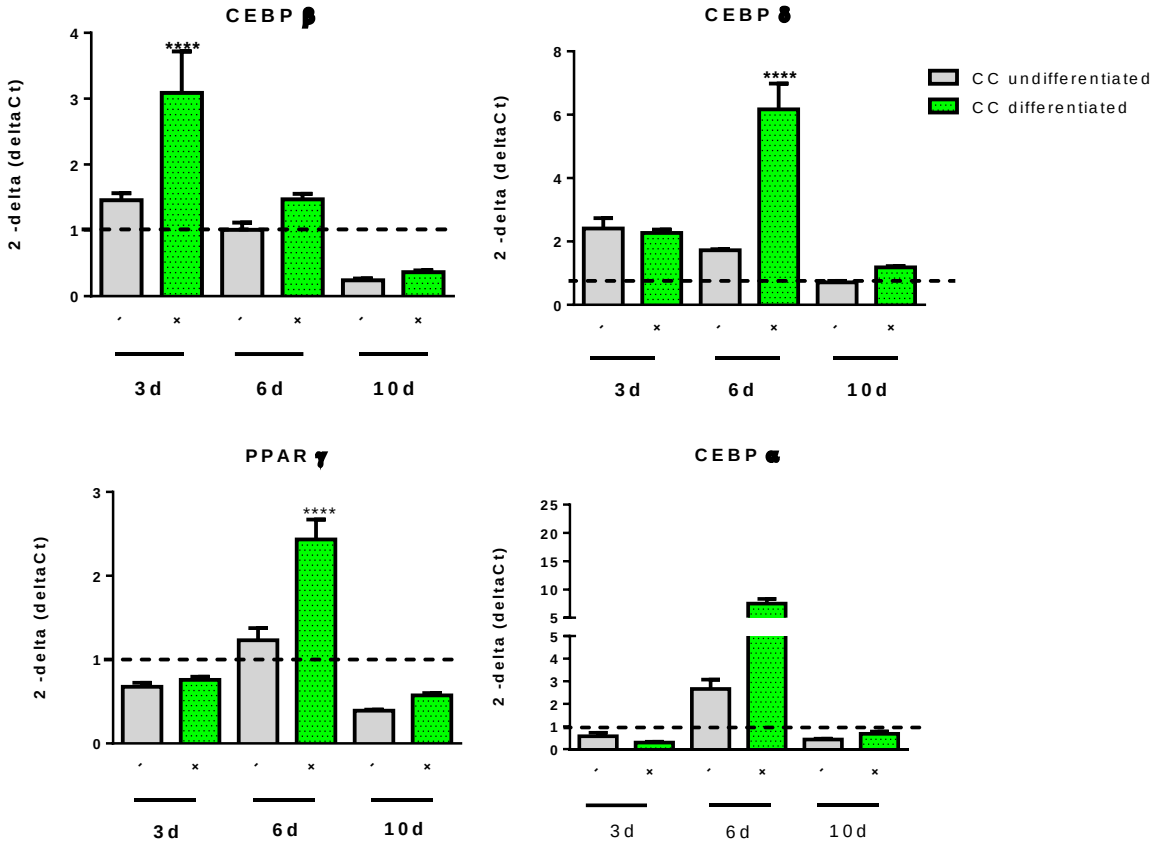

Figure 19. Effect of differentiation protocols on adipogenesis gene expression. Relative mRNA expression of nuclear-encoded genes CEBP beta, CEBP delta, CEBP alpha, and PPAR gamma in sw872 HTR2C CC stable clone. The expression of each gene was normalized to the expression of reference genes GAPDH and RPL32. Values are expressed as mean ± SEM (n ≥ 3 for each group)., **** *p < 0.0001 vs. T0*

DISCUSSION

SGAs are increasingly used in paediatric patients both in and off-label (Ho et al., 2011). The rise in SGA prescription rates may be due to the perception of improved safety compared with FGAs, especially related to a reduced risk for extrapyramidal side effects (Correll et al., 2004). However, SGA use is associated with rapidly increas(Whitehead et al., 2015)d appetite and weight gain which are the most frequent causes of loss of treatment adherence and reduced persistence in therapy, negatively affecting patients' health (Pozzi et al., 2016). Interestingly, these effects are not observed in all SGA-treated children, suggesting that some underlying genetic factors may predispose an individual to develop these adverse conditions (Lett et al., 2012) (Zhang et al., 2016).

Pharmacogenetic studies converge on several genetic variants that are reproducibly associated with weight gain induced by SGA treatment in the genes codifying for molecules involved in drug metabolism (i.e. CYP2D6) and for SGA receptors of the CNS that partly explain the inter-patient variability (Yoshida & Müller, 2019).

This project consists of two parts: an observational study on a cohort of paediatric patients and an in vitro experimentation on adipocytic cell line.

In the observational study, carried out in collaboration with four child neuropsychiatry centers, we have investigated the role of a panel of SNPs in genes to the pharmacodynamics of SGA and metabolic abnormalities. Among all factors studied, the rapid initial weight gain, antipsychotic drug administered, body mass index (BMI), and sex are the greatest predictors of weight gain and associated metabolic abnormalities (Gebhardt et al., 2009).

We aimed to find out which pharmacodynamics genetic variants may confer a risk for increased BMI-Z score; once established, these have the potential to identify individuals at increased risk to have weight gain.

To compare the subjects enrolled in the study, we considered as clinically significant an increase > or = 0.5 in BMI Z-score at 12 months (Wink et al., 2014). Hence, we have identified three different groups of patients (increase, decrease, stable BMI z score).

Unexpectedly, there were no significant differences in the three groups concerning the taken drug between risperidone and aripiprazole (BMI Z-score decrease vs increase *p=1.00;* BMI Z-score stable vs increase *p= 0.52*) Many clinical trials have shown that weight gain due to SGA, in particular risperidone, occurs predominantly during the first 4–6 months of treatment after which progressive

stabilization occurs (Calarge et al., 2012). This may support the existence of a plateau effect due to the time of exposure to risperidone.

Previous observations from clinical trials comparing risperidone and aripiprazole showed a similar end-point on weight gain, although the effect of aripiprazole seems to be delayed in time(Safer, 2004) (Mankoski et al., 2013). It is possible that we cannot appreciate this delayed effect of aripiprazole and the possible differences between the two drugs in terms of weight gain, because our patients are not naïve, but were already on treatment at baseline.

According to the value BMI-Z score, we classified our patients as normal, overweight, obese, and very obese. We observed the highest percentage of very obese/obese children at the last visit in the BMI-Z score increase group and the highest percentage of normal weight in the BMI Z-decrease group. The presence of polymorphisms that prevent the patient from gaining weight may be responsible for these differences in the population. However, since we do not have a complete picture of the patient's eating habits and lifestyle, we cannot completely rule out the idea that this relates to a change in the dietary plan.

To exclude a possible association between BMI-Z score changes and alteration in drug metabolism we first studied the SNPs in CYP2D6, the most relevant CYP isoform involved in the metabolism of risperidone and aripiprazole. The analysis of genotype is important to define the patients' phenotype through the definition of the Activity Score, which allows the grouping of individuals in the different phenotypic classes based on a score between 0 and 2.5. The distribution of phenotypic frequencies for each group shows an extremely high frequency of the NMs;, a very low frequency of UMs, and no PM patients, thus suggesting that the interpersonal variability of BMI change observed does not depend on differences in drug metabolism by CYP2D6.

The main limitation of this pharmacogenetic study is that CYP2D6 is considered as an isolated factor. Different enzymatic activity of CYP2D6 may contribute to interindividual variability in plasma drug concentration, and subsequently, clinical outcomes and different adverse effects (Jürgens et al., 2022). CYP2D6 is the main pathway of SGAs metabolism, but not the only one,as the drug elimination is often complex and involves several CYPs and other drug-metabolizing enzymes. These can affect drug plasma concentration and can impact on the interpretation of CYP2D6 genotype-predicted enzyme function.

From this point of view, the combination of pharmacogenetic analysis of metabolic enzymes and therapeutic drug monitoring can be the best strategy to get information about the clinical course in terms of drug efficacy and safety.
Then, we analyzed in our population the presence of polymorphisms in genes coding for receptors drug targets, that influence the pharmacodynamics of the drugs. The SNPs will be selected from a comprehensive meta-analysis by Zhang e al (Zhang et al., 2016) of associated gene variants with SGA-related weight gain. Between the 15 SNPs studied, the variants significantly associated with BMI changes are HTR2C rs6318, rs3813929, rs5181147, DRD2 rs1799732, rs6275, rs7131056 and MCR4 rs489693.

Regarding the serotonin receptor genes, the rs3813929 (C/T) promoter SNP (759C/T) of the X-linked HTR2C gene is one of the most consistent markers associated with weight gain. This variant is likely to have functional significance; the C allele has been consistently associated with increased odds in the risk of weight gain in meta-analyses examining multiple studies (Y. Chen et al., 2020). Reynold et al indicate that the C-759T polymorphism may affect transcription factor binding to the DNA, with the T allele associated with greater expression and less weight gain (Reynolds et al., 2003). Our results are in agreement with these data; the T- allele seems to be protective against weight gain ad more frequent in female patients with a BMI-Z score decrease.

The rs5818147(-697G/C) is in high linkage disequilibrium with rs3813929; Yuan et al. hypothesized their association with the obesity status and, specifically, of -697C, -759T alleles with leanness and showed a higher expression of the receptor in haplotypes containing both -759T and -697C alleles (Yuan et al., 2000). According to this hypothesis, the variants associated with a higher expression might have a protective effect against weight gain. In addition, the analysis of haplotypes by BR Godlewska and collaborators suggested that the presence of the -697C variant was crucial for the protective effect, and the -759T might have an additive effect (Godlewska et al., 2009).

Our results are consistent with those findings. In our patient's group, we observed a protective effect against SGA-induced weight gain in the case of both alleles, with a high frequency in BMI-Z score decrease female patients.

Contradictory results exist for the Cys23Ser polymorphism (rs6318). Basile and collaborators reported that the nonsynonymous polymorphism Cys23Ser of HTR2C showed a tendency, although not statistically significant, to be associated with clozapine-induced weight gain (Basile et al., 2001).

The only exception study was from Murashita et al (Murashita et al., 2005), which indicated a genetic susceptibility to weight gain in homozygous for the C minor allele. Our results are in agreement with this study; in hemizygotes males the presence of the C minor allele is associated with an increased BMI- Z score. In females, this association is only observed in heterozygosity (GC). Probably, the existence of different results may be explained by gender influence.

For DRD2 Lencz et al. have demonstrated that −141C deletion carriers (rs1799732) increased liability for SGA-induced weight gain when treated with olanzapine or risperidone (Lencz et al., 2010). The presence of the promoter region variation may produce fewer D2 receptors and the subsequent over-eating behavior and weight gain are exacerbated by the use of SGAs. Our results are in line with these observations; the presence of a deletion variant was associated with an increase in BMI-Z score in our population study.

In addition, Zhang et al have shown that the minor allele variants T in the SNP -rs6275 and A in rs7131056 may produce fewer DRD2 receptors, and subsequently, both are associated with the occurrence of weight gain (Zhang et al., 2016).

We observed discordant results only for rs6275 (939C); the C allele carrying patients gained more weight than G carriers. Probably these contradictory results are connected with other SNPs that interact with this rs6275. A common haplotype is rs6275 / rs6277 (957 T). The diminished stability of DRD2 transcript's and the impaired translation, with the consequent impact on weight gain, are most likely caused by the presence of both SNPs (Zhang et al., 2016).

MC4R rs489693 was found in association with SGA-related weight gain in a paediatric antipsychotic naïve cohort; the A minor allele homozygotes gained significantly more weight than C carriers (Malhotra et al., 2012). MCR4 in the hypothalamic paraventricular nucleus can induce decreased food intake and increased energy expenditure (J. D. Kim et al., 2014). Therefore, the MC4R gene has previously been identified as a candidate for weight-related phenotypes and mutations in this gene have been linked to extreme obesity in children and adolescents (Loos et al., 2008). Our results are in line with the literature, with the highest percentage of patients with AA homozygote genotype in the BMI-Z score increase group.

In summary, we conducted a preliminary association study of several candidate genes with BMI-Z score measures. We identified several genes that may be implicated in the development of BMI change in our paediatric patients.

Finally, the current investigation was limited by the number of observations, which were not systematically scheduled or taken in the same number for all patients, limiting study accuracy in

detecting changes. In the analysis, we omitted to consider several potentially relevant confounders, for example, familial and social characteristics, cognitive levels, concomitant lifestyle interventions, and others relative to the use of dietary and physical activity. Moreover, for future studies, it would be crucial to also include clinical data related to drug dose and pharmacokinetic analysis. While further work is required to confirm the interaction observed in this research, we consider that the identification of this SNP might provide useful input for personalized and individualized early intervention.

In the second part of this project, to examine the direct effect of SGAs and validate the role of selected SNP, at the peripheral level, we have performed in vitro experiment. The cellular and molecular mechanisms underlying the metabolic alteration associated with the consumption of antipsychotic drugs are not fully understood, but dysregulation in adipose tissue homeostasis has been suggested as a plausible explanation.

To investigate the impact of SGAs on dysfunctional adipose tissue we decided to use the SW872 liposarcoma cell model. This cell line is of human origin and that is an advantage for our study. Among the cell lines commonly proposed as adipocyte models, there is the 3T3-L1 preadipocyte murine cell line, which has been widely used for the study of obesity, diabetes and metabolism. Several comparative studies highlight significant differences in the genetic, metabolic, and physiologic character of adipose tissue among species. Considering these species-dependent variations, studies that aim to be translated into the clinic, like ours, should ideally be conducted on human cellular models of adipocytes (Kazantzis et al., 2012).

First, we performed a detailed characterization of the model to establish the cell culture condition for the adipogenic differentiation process. To this end, we treated SW872 with a specific cocktail that promotes the expression of transcriptional factors that control adipogenesis and inhibits cell growth (Fiorani et al., 2021). Adipogenesis was analyzed by comparing the lipid content and the expression of differentiation markers (CEBP alpha, CEBP beta, CEBP delta, and PPAR gamma) of cells cultured with the cocktail compared to those cultured with a standard growth medium at 3 - 6 - 10 days of culture. As expected, the lipid content and the differentiation markers expression were noticeably increased over time in treated cells.

Then, to clarify the mechanisms by which SGAs can induce weight gain, we treated our cells with risperidone, studying its effect on the adipogenesis of SW872. Notably, we observed that risperidone, administered to cells cultured in standard growth medium, directly promoted adipocyte differentiation and lipid accumulation. These results indicate that risperidone treatment may directly affect adipose

tissue independent of CNS and food intake, which is consistent with previous studies (Sárvári et al., 2014) (Vestri et al., 2007).

To better understand the phenotype of risperidone-induced adipocytes, next, we assessed the molecular expression levels of known brown adipocyte markers, PRMD16 and UCP1.

UCP1-mediated thermogenesis in brown adipose tissue (BAT) plays an important role in the regulation of energy expenditure. Furthermore, UCP1 is a major determinant of BAT thermogenic activity. Our data indicate that UCP1 and PRMD16 were reduced by risperidone treatment; these signals are consistent with the identification of a white adipocyte profile (Zheng et al., 2017). The shift from a white- to brown-adipocyte phenotype involves profound changes in the mitochondrial metabolic state. Expression and activation of UCP1 were followed by the increase in oxygen consumption, which was required to maintain ATP synthesis while uncoupling. In addition to higher uncoupling, this increased oxidative capacity in brown adipocytes relied upon active mitochondriogenesis (characterized by a higher mitochondrial DNA content), enhanced respiratory chain activity (characterized by increased maximal respiration and protein levels), and a modification in substrate preference towards fatty acids (28). Since mitochondrial respiration and uncoupling are hallmarks of brown adipocytes, we measured mitochondrial bioenergetic profiles to better understand the phenotype of risperidone-induced cells.

Risperidone treated adipocytes exhibited a decreased level of OxPhos ATP and ATP-linked respiration, moreover, basal respiratory capacity, as well as maximal respiration, showed a trend toward reduction. White adipocytes display a very low spare respiratory capacity and thus a poor ability to increase their mitochondrial activity. In contrast, the brown adipocytes are able to sharply increase their oxygen consumption and mitochondrial activity to respond to specific stimulation, and thus to increase uncoupling and perform thermogenesis (Chouchani et al., 2019). The capacity of risperidone treated adipocytes to reduce basal respiration and coupling activity confers a profile more similar to white adipocytes.

Total BAT activity depends on the rate of fatty acid oxidation, UCP1 expression and activity, and mitochondrial content. PGC-1α is a key transcriptional activator and master regulator of mitochondrial biogenesis (Prasun, 2020). It is likely to be the triggering factor that causes functional brown adipocyte differentiation.We found that PGC-1α is not enriched in adipocytes treated with risperidone, suggesting that they are destined to become white adipocytes. In line with this, our data also showed that *Hoxc4,* a key marker of white adipocytes, is highly expressed in risperidone adipocytes.

Overall, these results support the hypothesis that inhibition of brown adipogenesis and induction of white adipose tissue (WAT) adipocytes may be a possible mechanism by which risperidone induces weight gain as a side effect.

This observation is in line with clinical (Deng et al., 2010) and animal studies (Sylvester et al., 2020) that showed that body weight gain under risperidone treatment was due to a specific accumulation of white adipose tissue. Our results indicate that the adipose tissue may be a key target of antipsychotics for their undesirable side effects on the regulation of energy storage and expenditure. Further research is necessary to weigh the pathophysiological significance of our *in vitro* findings in humans.

Finally, we have developed a complete workflow for the insertion of SNPs identified in our population study as related to BMI changes after SGAs administration (DRD2 rs6275; HTR2C rs6318). Our workflow starts with site-direct mutagenesis of the genes cloned in an expression vector to introduce the SNP, followed by transfection of SW872 and screening and clonal expansion of cells stably transfected. Using our adipocyte differentiation protocol, we further showed that stably transfected clones could be differentiated into adipocytes, therefore providing a relevant model for studying the SNP role in SGAs-induced adipogenesis. This model may allow us to understand if the BMI changes observed in our patients are related to an SNP modification in the receptor's mechanism of action at the peripheral level.

CONCLUSION

In conclusion with this project we have identified several genes that may be implicated in the development of BMI change in our paediatric patients in treatment with SGAs. These data complemented with the evaluation of plasmatic concentration of the drugs and their active metabolites, will contribute to define therapeutic windows/reference ranges for paediatric patients, still not available.

To examine the direct effect of SGAs and validate the role of these SNP, at the peripheral level, we have performed in vitro experiment in the SW872 cell line.

Our data revealed that risperidone promotes adipocyte differentiation and seems to induce a profile more similar to white. This might be a possible mechanism by which risperidone induces weight gain as a side effect.

Finally, we have designed an in vitro model system in which we obtained the introduction of selected SNPs, present in our cohort of pediatric patients,.

The design of a cellmodel expressing the SNPs identified in our cohort of paediatric patients will allow us to examine their role in metabolic pathways involved with obesity.

REPORT

Second generation antipsychotics (SGAs) are increasingly used in pediatric patients both in- and off-label (Ho et al., 2011). The rise in SGA prescription rates may be due to the perception of improved safety compared with first generation antipsychotics, especially related to a reduced risk for extrapyramidal side effects (Correll et al., 2004). However, SGA use is associated with rapid weight gain and adverse drug reaction (ADR) such as metabolic syndrome (3). Interestingly, these side effects are not observed in all SGA-treated children, suggesting some underlying genetic factors may predispose an individual to develop these adverse conditions (Lett et al., 2012) (Zhang et al., 2016).

Genome wide studies have been developed to identify SNPs in the molecules involved in drug metabolism (CYP450) (Lett et al., 2012) and in genes for SGA receptors of the central nervous system (Reynolds et al., 2003)(Lencz et al., 2010) that 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 metabolic ADRs. The aims of this are:

Aim 1: **Evaluate the association of SNPs found in the selected in genes** with the occurrence of ADRs **and metabolic imbalances** in a cohort of pediatric patients

Aim 2: **Correlate the safety of SGAs** in this group of children with the pharmacological dose, the plasma concentrations and the pharmacogenetics

Aim 3: Validate in **adipocyte- and enterocyte-**like cell lines the **impact of SNPs** on differentiation (adipocytes) and release of factors contributing to metabolic regulation.

These aspects are evaluated in a clinical study in collaboration with four child neuropsychiatry centers in which 200 pediatric patients, affected by disruptive behavioral disturbances, who have been prescribed SGAs, have been enrolled. Patients have been subjected to routine outpatient procedures with controls every 3 months. The blood has been collected for: routine analyses, analysis of biomarkers of metabolism, and pharmacogenetic analysis and monitor patients' clinical course. The manifestations of metabolic adverse events to the administered drug, have been analysed by

pharmacovigilance studies. The biological role of candidate SNPs in modulating triggering factors associated with metabolic regulation have been validated in in vitro systems.

The recruitment of patients started in January 2019. So far, 200 pediatric patients have been enrolled.

We performed our analysis on 150 of them. Most patients are male (70.4%) with a mean age of 13 years and are in treatment with risperidone (73.1%) or aripiprazole (63.3%). Regarding the disease, most patients are autistic but psychiatric diagnosis also included schizophrenia, psychosis, anxiety disorders, ADHD and bipolar disorders. All data regarding patients are collected in a unique dedicated database, in order to study possible correlation between presence of SNPs and adverse events during the pharmacological treatment.

We first analysed ADRs occurring with SGAs in order to define which are the most common and understand if our population is a good model for our study. The data, obtained from a pharmacovigilance analysis, showed that, ADRs were 37 and mainly associated with aripiprazole therapy; among these, 56% of ADRs are cardiometabolic events.

The International Diabetes Federation (IDF) suggests that for children aged 10 years or older, metabolic syndrome can be diagnosed with abdominal obesity and the presence of two or more other clinical features (*i.e.* elevated triglycerides, low HDL-cholesterol, high blood pressure, increased plasma glucose) (Zimmet et al., 2007).

We analysed the changes of these anthropometric metabolic parameters over time in our population study (Figure 1).

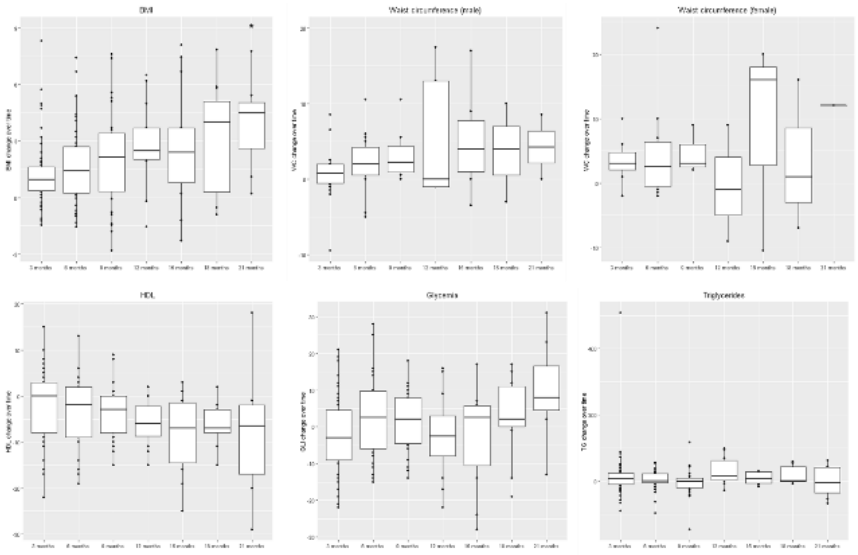

Figure 1 Analysis of metabolic syndrome anthropometric parameters over time. Upper panels from left to right: BMI, Waist circumference (males), Waist circumference (females). Lower panels from left to right: Cholestrol-HDL; Glycemia, Triglycerides

We observed a significant difference only for BMI and waist circumference in male patients after 21 weeks from the enrolment, and in general an upward trend for all parameters over time.

In the next step we will study the prevalence of MetS in our population according to the diagnostic criteria and through logistic regression analysis we will test which of the parameters is the strongest predictor of MetS.

For pharmacogenetic analyses, we first studied SNPs in CYP2D6, the most relevant CYP isoform involved in the metabolism of risperidone and aripiprazole (Youngster et al., 2014).

The CYP2D6 allelic variants that we have analyzed for all patients are reported in Table 1.

Gene	Polymorphism	
CYP450 2D6	*2 (2850C>T)	*9 (2615_2617delAAG)
	*3 (2549delA)	*10 (100C>T)
	*4 (1846G>A)	*12 (124G>A)
	*5 (CYP2D6 deleted)	*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)

Table 1. CYP2D6 polymorfisms analysed by the INFINITI CYP450 2D6I Assay – Auto Genomics microarray (from datasheet).

The analysis of genotype is important to define the patients phenotype through the definition of the Activity Score which allows the grouping of individuals in the different phenotypic classes on the basis of a score between 0 and 2.5 (Crews et al., 2021). As expected, we found in our population a very high frequency of the normal metabolizer phenotype (65%). The intermediate metabolizer phenotype is present in 26,7% of the samples analyzed while individuals with ultrarapid, poor and intermediate-normal phenotype reach frequencies of 4.67%, 1.3% and 1.3%, respectively. These data suggest that the interpersonal variability in terms of metabolic response observed after SGA administration may depend on the presence of polymorphisms in the genes coding for molecules that influence the pharmacodynamics of SGAs that are highly associated with food intake, metabolism, and body weight (Zhang et al., 2016). To this end, we analyzed these variants in our study population (Table 2).

Gene	Variant	Gene	Variant
ADRA2A	rs1800544 (c.-1252G>C)	HTR2C	rs6318 (c.68G>C)
ADRB3	rs4994 (c.190T>C)	HTR2C	rs518147 (c.-697C>G)
BDNF	rs6265 (c.196G>A)	HTR2C	rs1414334 (c.551-3008C>G)
DRD2	rs1799732 (c.-486_-485insC)	INSIG2	rs17047764 (g.118868582G>C)
DRD2	rs6275 (c.939T>C)	MCR4	rs17782313 (g.60183864T>C)
DRD2	rs7131056 (c.-32+16024T>G)	MCR4	rs489693 (g.60215554C>A)
DRD2	rs1799978 (c.-585A>G)	SNAP25	rs1051312 (c.*243T>C)
HTR2A	rs6313 (c.102C>T)	DRD3	rs6280 (c.25G>A)
HTR2C	rs3813929 (c.-759C>T)	HTR6	rs1805054 (c.267C>T)

Table 2. SNPs in genes of SGAs pharmacodynamics.

A preliminary association analysis for these SNPs was performed showing that the observed allelic frequencies are statistically in equilibrium with the Hardy-Weinberg law ($p > 0.05$).

After genotyping all the patients, an assessment of the association of genotypes of the studied polymorphic variants with a pathological metabolic phenotype will be carried out using the odds ratio (OR). This will allow 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 define a model of personalized medicine based on the individual characteristics of each patient.

Another goal of this project is to define the role of polymorphisms of the genes involved in the mechanisms of action of SGAs in peripheral tissue by performing in vitro experiments. In particular, we focused our attention on polymorphisms in the genes for serotonin and dopamine receptors, in one cell models: SW872 (human liposarcoma cells).

We started our experiment with the SW-872 liposarcoma cell line and selected as SGA, risperidone.

First, we performed a detailed characterization of the model establishing the cell culture condition for adipogenic differentiation process. To this end we treated SW-872 with a specific cocktail that promotes the expression of transcriptional factors that control adipogenesis and inhibits cell growth (Fiorani et al., 2021) Adipogenesis was analyzed by comparing the lipid content and the expression of differentiation markers (CEBP alpha, CEBP beta, CEBP delta and PPAR gamma) of treated and untreated cells at 3 - 6- 10 days of culture. The lipid content of treated cells, measured by the Oil-red staining, was noticeably increased over time. For differentiation markers expression we observed a significant increase starting from day 6 of treatment.

Then, in order to clarify the mechanisms by which risperidone induces weight gain (Rojo et al., 2015), we studied its effect on adipogenesis of SW-872, by treated them with a dose that did not affect cell viability for 3 – 6 – 10 days. As positive control of differentiation we used the cells treated with the differentiation cocktail and as negative control cells treated with vehicle alone. We observed that risperidone promoted lipid accumulation with a significant increase at day 6 compared to untreated cells. Similar results were found for differentiation markers expressions which increased significantly after risperidone treatment. Further experiments will be carried out to define the possible molecular mechanism of risperidone-induced adipocyte differentiation in this cell model.

Finally, we designed the system for the introduction of SNPs in our cells by choosing the site directed mutagenesis approach (H. Han et al., 2020). We selected two SNPs: rs6318 for the 5-HT2C gene and rs6275 for the DRD2 gene, already study in correlation with weight gain, metabolic ADRs and antipsychotic treatment with a focus on SNC expression and also observed in our population.

The two variant alleles for each gene were subcloned into a neomycin-resistant pcDNA3.1(+) _myc tag - vector and transfected in our vitro models. SW-872 stably transfected cells were selected in medium supplemented with 500 µg/mL neomycin and the proteins were visualized by confocal microscopy after the staining with anti-Myc antibody. Some selected clones were characterized through RT-PCR and Western Blot in order to assess the transcript and the protein expression level. The clones with increased levels of protein expression were selected for future experiments. New

experiments are ongoing to evaluate whether the presence of the SNPs can modify the adipogenic differentiation process in the presence or absence of risperidone.

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CONGRESS PARTICIPATION

- Relatore durante **40° CONGRESSO NAZIONALE DELLA SOCIETÀ ITALIANA DI FARMACOLOGIA** - Il valore scientifico e l'uso appropriato del farmaco;con relazione dal titolo: Pharmacogenetics of antipsychotic drugs, Marzo 2021
- Speaker durante il workshop "Nuovi modelli in vitro e in vivo per studiare i tumori: dagli organoidi agli organismi superiori" presso la Fondazione IRCCS Istituto Nazionale dei Tumori di Milano 2018 con presentazione dal titolo "Organoidi dal carcinoma ovarico: sviluppo del modello più adatto allo studio di questo tumore
- Napoli, F. Roggiani, M. Bagnoli, F. Raspagliesi, A. Invernizzi, A. Tomassetti, D. Mezzanzanica. Heterogeneity of ascites from epithelial ovarian cancer: from cells to microenvironment. Abstract "**60th MEETING OF THE ITALIAN CANCER SOCIETY,** Milano", 2018.
- M. Bonzanni, A. Napoli et al. Effects of miRNAs modulated by endurance training on cardiomyocyte excitability. Abstract "**67th Meeting of the Italian Society of Physiology**, Catania, 2016

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