Running Title: FXR1/GSK3β signaling pathway and Negative Symptoms.

Title: Evidence of an interaction between FXR1 and GSK3β polymorphisms on levels of Negative Symptoms of Schizophrenia and their response to antipsychotics

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Abstract

**Introduction:** Genome Wide Association Studies (GWAS) have identified several genes associated with schizophrenia (SCZ) and exponentially increased knowledge on the genetic basis of the disease. Additionally, products of GWAS genes interact with neuronal factors coded by genes lacking association, such that this interaction may confer risk for specific phenotypes of this brain disorder. In this regard, *FXR1* (Fragile-X mental-retardation-syndrome-related 1) gene has been GWAS associated with SCZ. *FXR1* protein is regulated by Glycogen Synthase Kinase-3β (*GSK3β*), which has been implicated in pathophysiology of SCZ and response to Antipsychotics (APs). rs496250 and rs12630592, two eQTLs of *FXR1* and *GSK3β* respectively, interact on emotion stability and amygdala/PFC activity during emotion processing. These two phenotypes are associated with Negative Symptoms (NS) of SCZ suggesting that the interaction between these SNPs may also affect NS severity and responsiveness to medication.

**Methods:** To test this hypothesis, in two independent samples of patients with SCZ, we investigated rs496250 by rs12630592 interaction on NS severity and response to APs. We also tested a putative link between APs administration and *fxr1* expression, as already reported for *GSK3β* expression.

**Results:** We found that rs496250 and rs12630592 interact on NS severity. We also found evidence suggesting interaction of these polymorphisms also on response to APs. This interaction was not present when looking at positive and general psychopathology scores. Furthermore, chronic olanzapine administration led to a reduction of *FXR1* expression in mouse frontal cortex.

**Discussion:** Our findings suggest that, like *GSK3β*, *FXR1* is affected by APs while shedding new light on the role of the FXR1/GSK3β pathway for NS of SCZ.

**Keywords:** Schizophrenia; Negative Symptoms; FXR1; GSK3β; Treatment with Antipsychotics.
1. Introduction

Genome Wide Association Studies (GWAS) identified hundreds of low penetrance genetic loci involved in risk for Schizophrenia (SCZ) (1, 2). GWAS alleles clustering to specific biological pathways may underlie specific illness phenotypes (3-5). However, risk genes also interact with genes that, though not surviving statistical thresholds of Genome Wide association, may have a role in the pathophysiology of SCZ, thus potentially impacting on the full biological manifestation of risk (6).

Among genetic loci associated with SCZ by GWAS, FXR1 codes for fragile X mental retardation syndrome-related protein 1 (FXR1P), a RNA binding protein related to the fragile-X mental retardation protein (FMRP) (1, 7). FXR1P is known to interact with FMRP (8, 9) and large-scale genetic studies have consistently indicated involvement of FMRP targets in the genetic architecture of SCZ (10, 11). Furthermore, molecular studies have demonstrated that FXR1P is potentially regulated by dopamine receptor (DAR) (12, 13) and regulates ionotropic Glutamate Receptor (13). Both type of receptors that have robustly been implicated in the pathophysiology of SCZ and mechanism of action of Antipsychotic (AP) medication (14, 15). However, whether FXR1P can be modulated by APs has not been demonstrated.

In a previous study (12), we have demonstrated a functional interaction between FXR1P and the Glycogen Synthase Kinase 3 beta (GSK3β). This kinase phosphorylates FXR1P and facilitates its degradation in neurons. Importantly, the GSK3β gene has been consistently implicated in the modulation of SCZ-related phenotypes (16), along with response to APs (17-19). Furthermore, analysis of postmortem brains have showed decreased GSK3β phosphorylation and protein levels in frontal cortex or lower GSK3β mRNA levels in dorsolateral prefrontal cortex (DLPFC) of SCZ as compared to healthy individuals (20-22). GSK3β is a known effector of Type 2 Dopamine Receptor (DRD2) signaling (23-25). DRD2 has been involved in the pathophysiology of SCZ (26-29) and is the main molecular target of AP medication (30, 31). In addition, the
contribution of GSK3β to AP response has been also related to alternative molecular pathways not
directly involving DRD2 and dopamine neurotransmission as a whole, such as those related to
Wnt pathway, glutamate receptors and serotonin receptors (22, 32, 33).

We identified two SNPs associated with postmortem Prefrontal Cortex FXR1 and GSK3β
mRNA expression rs496250 and rs12630592, that have a combined effect on behavioral and brain
phenotypes related to the processing of emotions (12). More specifically, the interaction between
the rs496250 and rs12630592 SNPs in healthy subjects is associated with Emotional Stability, as
defined within the Big Five Personality Trait model, as well as with amygdala activity during an
emotion recognition task. These variants may also affect symptom severity in bipolar disorder
(34).

Emotional Stability and amygdala activity during emotion processing are linked with
Negative Symptoms (NS) of SCZ (35-39), a core clinical domain of the disorder at least partially
heritable (40, 41) and associated with genetic variation by both candidate gene approaches and
Genome Wide Association Studies (42-47). This suggests that the GSK3β-FXR1- signaling
module and related genetic variation affecting GSK3β and FXR1 expression levels may be
involved in brain and clinical phenotypes related to NS, potentially including response to AP
treatment. On this basis, we investigated the interaction between rs496250 and rs12630592
functional variations within FXR1 and GSK3β (12, 16) on NS severity and response to AP in
patients with SCZ.

Furthermore, we investigated putative modulation of FXR1 by AP - as already reported for GSK3β
(17-19) - by studying the effect of chronic administration of the second generation AP olanzapine
on mouse frontal cortex fxr1 gene expression.

We hypothesized that rs496250 and rs12630592 interact on NS severity and response to
AP in patients with SCZ and that olanzapine administration is associated with fxr1 expression in
mouse frontal cortex.
2. Methods and materials

2.1. Experiments in humans

Samples

Discovery Sample

We pooled data from two independent samples (Sample 1 and Sample 2) into a single

Discovery Sample (DS) in order to maximize our sample size and reduce Type I errors.

Sample 1 included 266 patients with SCZ or Schizoaffective disorder (201 males; Mean
Age: 35.9±SD =10) recruited in the region of Apulia, Italy. Recruitment procedures were carried
out in accordance with The Code of Ethics of the World Medical Association (Declaration of
Helsinki) and approval was given by the local ethics committee (‘‘Comitato Etico Indipendente
Locale - Azienda Ospedaliero-Universitaria Consorziale Policlinico di Bari’’). Diagnosis of SCZ
was made using the Structured Clinical Interview for the DSM-5, Axis 1 disorders (Diagnostic
Publishing, 2013), which was administered by psychiatrists. Patients were excluded if they had: a
significant history of drug or alcohol abuse; active drug abuse in the previous year; experienced a
head trauma with a loss of consciousness; or if they suffered from any other significant medical
condition. NS were assessed at study entry (T0) and at day 28 (4 weeks or T1) with the PANSS.
Such a scale was administered by a trained psychiatrist, who was blind to FXR1 rs496250 and
GSK3β rs12630592 genotype.

Patients were treated for 4 weeks with an AP therapy (Mean AP stable dose = 574,9 mg
Chlorpromazine Equivalents). More in detail, the majority of patients underwent
monotherapy with Olanzapine (73 out of 266). Other interventions included Risperidone,
Clozapine, Quetiapine, Aripiprazole, Paliperidone and Haloperidol. 51 out of 266 patients
received more than one AP, 20 out of 266 underwent concomitant medication with
antidepressants, while 51 out of 266 concomitant medication with mood stabilizers.
Sample 2 included a subgroup of individuals recruited within the CATIE Study (48). Characteristics of the CATIE sample are described elsewhere (49). For the purpose of the current study, 121 subjects with diagnosis of SCZ according to DSM-4 having full genetic and clinical information were studied (91 males; Mean Age: 38.9 ±SD =11.5). As in sample 1, NS were assessed at the study entrance (T0 or study baseline) and one months later (T1) (mean AP stable dose = 479.8 mg Chlorpromazine Equivalents). In detail, patients underwent treatment with Olanzapine, Quetiapine, Risperidone, Perphenazine and Ziprasidone and overlap between different antipsychotics was permitted only for the first four weeks after randomization. Concomitant medications were allowed throughout the trial, except for additional antipsychotic agents. 61 out of 148 patients in sample 2 underwent concomitant medications with antidepressants.

Replication Sample

The Replication Sample included 116 patients with SCZ and schizophreniform disorder (49 males; Mean Age±SD: 39 ± 12.85) recruited at the University of Brescia who satisfied criteria of DSM-5 (50). Subjects underwent monotherapy with Olanzapine (N= 58) or Risperidone (N=58). Changes in symptom severity were monitored by administering the PANSS scale at the study entrance (T0 or study baseline) and after two weeks of stable treatment (T1).

2.2. Genotyping

Sample 1

FXR1 rs496250 and GSK3β rs12630592 genotypes in sample 1 were ascertained using an Illumina HumanOmni2.5-8 v1 BeadChip platform. More in the detail, approximately 200 ng DNA was used for genotyping analysis. DNA was concentrated at 50ng/ml (diluted in 10 mM
Tris/1mM EDTA) with a Nanodrop Spectrophotometer (ND-1000). Each sample was whole-genome amplified, fragmented, precipitated and re-suspended in appropriate concentrations of hybridization buffer. Denatured samples were hybridized on the prepared Illumina HumanOmni2.5-8 v1 BeadChip. After hybridization, the BeadChip oligonucleotides were extended by a single labeled base, which was detected by fluorescence imaging with an Illumina Bead Array Reader. Normalized bead intensity data obtained for each sample were loaded into the Illumina GenomeStudio (Illumina, v.2010.1) with cluster position files provided by Illumina, and fluorescence intensities were converted into SNP genotypes. After genotypes were called and the pedigree file was assembled, we removed SNPs showing minor allele frequency < 1%, genotype missing rate > 5%, or deviation from Hardy-Weinberg equilibrium (p < 0.0001). Individuals were also removed if their overall genotyping rate was below 97%. Sample duplications and cryptic relatedness were ruled out through identity-by-state (IBS) analysis of genotype data.

**Sample 2**

Genotyping procedures for sample 2 are described elsewhere (49).

Because of the low Minor (A) Allele Frequency (MAF) of rs496250, in all analyses A-homozygote subjects (AA) were collapsed with heterozygotes (AG) as in a previous report (12). Genotype composition of our samples is described in Table 1.

**Replication sample**

Genotyping procedures for Replication Samples are described elsewhere (50).

Furthermore, since in this cohort the number of minor allele carrier individuals were extremely small (FXR1 rs496250 AA/GSK3β rs12630592 GG = 1, FXR1 rs496250 AA/GSK3β rs12630592 GT = 0, FXR1
rs496250 AG/GSK3β rs12630592 GG = 16, FXR1 rs496250 AG/GSK3β rs12630592 GT = 14,
FXR1 rs496250 AG/GSK3β rs12630592 TT = 7), within the following statistical analyses we
collapsed individuals with FXR1 rs496250 AA and AG genotypes in a single “A-carriers”
group, and GSK3β rs12630592 TT and AT genotypes in a single “T-carriers” group.

Genotype composition of the Replication Sample is provided in Table 1.

2.3. Statistical Analyses

2.3.1. Sample 1 and 2 pooling

Before pooling sample 1 and sample 2 into the DS, the two samples were investigated for
putative differences in age, gender, PANSS Negative Scores at study baseline, dose of APs as
converted to Chlorpromazine Equivalents (CE) (51). One-way ANOVAs using sample as the
independent variable and either age, PANSS Negative Scores or CE AP dose as the dependent
variable were used to assess sample matching as for these variables. A Pearson’s chi-squared test
was used to check for gender matching between the two samples. Moreover, in order to further
control for inter-sample heterogeneity, each individual was given a factor level dichotomous
variable (Sample Factor, or SF) indicating the sample s/he belonged to and SF was introduced as
covariate of no interest in all statistical analyses (52).

ANOVA revealed that age was lower in sample 1 than in sample 2 (p<0.003), PANSS
Negative Scores were higher in sample 1 than in sample 2 (p<0.0001), and mean stable dose of
APs expressed in CE was higher in sample 1 than in sample 2 (p=0.0005). No statistically
significant difference was observed across gender distribution in the two samples (p>0.05).

Therefore, age, gender, CE and SF were introduced as covariates of no interest in the
statistical model. Furthermore, we used genome-wide genotypes to compute genomic
eigenvariates, which afford a multidimensional representation of ancestry by means of
singular value decomposition applied to allelic count at each polymorphic locus considered.

We thus obtained, within each dataset we used for our analyses, a set of variables
representative of population stratification. Both the cohort we recruited in Bari for the current study (sample 1) and the CATIE sample (sample 2) included Caucasian ancestry male and female participants; hence, genomic eigenvariates in these samples indexed a relatively restricted range of population stratification.

More in the detail, we computed genomic eigenvariates by performing a Principal Component Analysis separately for each of the two cohorts using SNPs with high imputation quality (INFO >0.8), low missingness (<1%), MAF>0.05 and in relative linkage equilibrium (LD) after 2 iterations of LD pruning (r² < 0.2, 200 SNP windows). We removed long-range-LD areas (MHC and chr8 inversion).

2.3.2. Effect of FXR1 rs496250 and GSK3β rs12630592 genotypes and their interaction on NS
We performed a factorial ANOVA to investigate the main effect of FXR1 rs496250 and GSK3β rs12630592 genotypes and their interaction on NS, with the genotypes of interest as independent variables and the PANSS NS Score after one month of stable dose of AP treatment (T1, or day 28 for sample 1, and visit 1 for sample 2), as the dependent one. Potential confounding effects of population stratification were corrected for by marginalizing the PANSS NS Score for the first 5 principal genomic eigenvariates, separately for each cohort. Standardized residuals were computed by performing linear regression analysis with the first 5 principal genomic eigenvariates as independent variables, and the PANSS NS Score as the dependent variable. Site-specific standardized residuals were then used for the analysis. To provide further confirmation of results, analogous analyses were performed in sample 1 and sample 2 of the DS separately with the same statistical approach described above (See Supplementary Material).

Confirmatory analysis was performed on the Replication Sample by using the same
statistical approach. Principal genomic eigenvariates were computed as described for the DS. Chlorpromazine equivalents were not used as covariates in this analysis, because they were not available in this sample.

Finally, in order to assess the specificity of rs496250 and rs12630592 effects on NS, similar analyses were performed on Positive and General Symptoms of SCZ, respectively measured with the “Positive” and “General” subscales of the PANSS.

All post-hoc analyses were performed using Fisher’s test. Based on our strong a priori hypothesis on the effects of rs496250 and rs12630592 on phenotypes of interest based on the DS results, one-tailed statistics was used in post-hoc analyses on the Replication Sample.

2.3.3. Effect of FXR1 rs496250 and GSK3β rs12630592 genotypes and their interaction on Negative Symptom response to APs

Response to APs in terms of NS was measured as the variation of PANSS Negative Scores from T0 to T1 that we indicated as Δ-N-PANSS. In order to establish the main effect of FXR1 rs496250 and GSK3β rs12630592 genotype and their interaction on Δ-N-PANSS, we performed a Factorial ANCOVA, with FXR1 rs496250 and GSK3β rs12630592 genotypes as independent variables and Δ-N-PANSS as the dependent one. Since response to APs could be affected by severity of NS at the study entry (T0) and by the stable dose APs subjects were assuming, we normalized the Δ-N-PANSS to PANSS Negative scores at T0.

Again, potential confounding effects of population stratification were corrected for by marginalizing the Δ-N-PANSS for the first 5 principal genomic eigenvariates, separately for each cohort. Standardized residuals were computed by performing linear regression analysis with the first 5 principal genomic eigenvariates as independent variables, and the Δ-N-PANSS as the dependent variable. Site-specific standardized residuals were then used for the analysis.
Because of study discontinuation, 183 out of 387 patients in the DS (males 121; mean age $= 29.4 \pm SD = 8.2$) who entered the study were assessed at T1 and were available for Δ-N-PANSS computation.

Confirmatory analysis using the same statistical approach was performed on the Replication Sample by using the same statistical approach. Principal genomic eigenvariates were computed as described for the DS. Chlorpromazine equivalents were not used as covariates in this analysis, because they were not available in this sample.

Moreover, to provide further confirmation of results, analogous analyses were performed in sample 1 and sample 2 separately with the same statistical approach described above (See Supplementary Material).

Finally, in order to assess the specificity of rs496250 and rs12630592 effects on NS, similar analyses were performed on Positive and General Symptoms of SCZ, respectively measured with the “Positive” and “General” subscales of the PANSS.

All post-hoc analyses were performed using Fisher’s test. Based on our strong a priori hypothesis on the effects of rs496250 and rs12630592 on phenotypes of interest based on the DS results, one-tailed statistics was used in post-hoc analyses on the Replication Sample.

2.4 Animal experiments

2.4.1. Animals

Ten week old C57BL/6J mice were used for current experiment. All mice were housed individually in controlled 12hr light/12hr dark cycle, constant temperature and humidity environment. No changes in corncob layer were made during the entire experimental period. All animals in the experiment were drug naïve and were used only for a single experiment. All animal procedures were performed in accordance with the Canadian Council of Animal Care guideline and following formal approval by the University of Toronto Animal Ethics Committee.
2.4.2. Treatment

The activity of GSK3β has been shown to be affected by APs in several experimental settings (20, 53). To verify whether FXR1P can also be affected by AP drugs, mice were treated with olanzapine for 30 days in chow. Mice were randomly assigned to two different arms of treatment (ten mice for each arm), one olanzapine-treated and the other one vehicle-treated. 54 mg/kg concentration pure olanzapine administered to animals in chow. Olanzapine dose was adjusted in order to reach a steady-state plasma level (21±5 ng/ml) closed to previously reported (54) clinically relevant range (10–50 ng/mL). Chow without olanzapine was used as vehicle.

2.4.3. Tissue Dissection

Mice were sacrificed after 30 days of treatment by rapid cervical dislocation. Brains were dissected on an ice-cold surface. Prefrontal cortex (PFC) 500 nm thick serial coronal sections were prepared using ice-cold adult mouse brain slicer and matrix (Zivic instruments) and PFC was sectioned with microsurgical knife. Finally, samples were stored at -80°C until analysis.

2.4.4. PFC RNA extraction and qPCR

Impact of treatment on FXRI expression in the prefrontal cortex was evaluated using quantitative PCR. Total RNA was extracted from mouse PFC using Direct-zol RNA MiniPrep (Zymo Research) and converted into cDNA using SuperScript IV VILO Master Mix synthesis system (Invitrogen #11756050) according to manufacturer’s instructions. qPCR analysis was performed according to TaqMan Fast Advanced Master Mix protocol on a QuantStudio3 Real-Time PCR System (Thermo Fisher Scientific) using Thermo Fisher Scientific Mm00484523_m1 FXRI probe and Thermo Fisher Scientific Mm99999915_g1 GAPDH probe as internal control. Relative expression quantification analyses were carried out on biological triplicates of each sample on a QuantStudio TM Design and Analysis Software
Mean Ct values of \( FXR1 \) were normalized to those of \( GAPDH \). These normalized values were analysed through the comparative Ct Method for the relative quantification of targets as previously reported (55).

### 2.4.5. Statistical analysis

A one-way Analysis of Variance (ANOVA) with \( FXR1 \) gene expression level as the dependent variable and treatment arm (olanzapine vs vehicle) as the independent variable was performed in order to establish the impact of olanzapine as compared to vehicle on \( FXR1 \) expression.

### 3. Results

#### 3.1. Interaction of \( FXR1 \) rs496250 and \( GSK3\beta \) rs12630592 on Negative Symptom severity

In the DS, factorial ANOVA on Negative Symptom severity indicated no main effect of \( FXR1 \) rs496250 and \( GSK3\beta \) rs12630592 genotypes (all \( p \)-values >0.05), while their interaction was significant (\( F=3.11; \ p=0.045 \) (Figure 1). Fisher’s post-hoc analyses showed that, in the context of \( FXR1 \) rs496250 A-carrier genotype, subjects carrying rs12630592 GG genotype have higher N-PANSS compared with rs12630592 TT (\( p=0.005 \)) subjects. Furthermore, \( FXR1 \) rs496250 A-carrier/rs12630592 GG subjects have higher N-PANSS than \( FXR1 \) rs496250 GG/rs12630592 GG subjects (\( p=0.02 \)), \( FXR1 \) rs496250 GG/rs12630592 GT subjects (\( p=0.03 \)) and \( FXR1 \) rs496250 GG/rs12630592 TT subjects (\( p=0.04 \)).

Similar analyses on the Replication Sample indicated consistent results with those obtained on the DS. In detail, we found that \( FXR1 \) rs496250 and \( GSK3\beta \) rs12630592 genotypes interacted on NS severity (\( F=4.3; \ p=0.04 \) (Figure 2). Fisher’s one-tailed post-hoc analyses showed that, in the context of \( FXR1 \) rs496250 A-carrier genotype, subjects carrying rs12630592 GG genotype have higher PANSS NS scores compared with rs12630592 T-
carrier (p= 0.045) subjects. Furthermore, rs12630592 GG/\(FXRI\) rs496250 A-carrier subjects have higher PANSS NS scores than \(GSK3\beta\) rs12630592 GG/\(FXRI\) rs496250 GG subjects (p = 0.025).

Separate analyses on sample 1 and 2 indicated consistent results with those obtained with the pooled Sample 1 and Sample 2 (see Supplementary Material).

No main effect of \(FXRI\) rs496250 and \(GSK3\beta\) rs12630592 genotypes, nor rs496250-by-rs12630592 interaction was observed on the PANSS “Positive” and “General” subscale scores (all p-values >0.05).

3.2. Interaction of \(FXRI\) rs496250 and \(GSK3\beta\) rs12630592 on Negative Symptom response to APs

In the DS, factorial ANCOVA showed no main effect of \(FXRI\) rs496250 and \(GSK3\beta\) rs12630592 genotype on \(\Delta\)-N-PANSS (all p-values >0.05). Nonetheless, the same analysis indicated a significant interaction between rs496250 and rs12630592 on \(\Delta\)-N-PANSS (F=3.3; p=0.05) (Figure 3). Post-hoc analyses indicated that, in the context of \(FXRI\) rs496250 A-carrier genotype, subjects with rs12630592 TT genotype have higher \(\Delta\)-N-PANSS compared with both rs12630592 GT (p= 0.003) and rs12630592 GG (p= 0.006) genotype. Statistically significant difference in \(\Delta\)-N-PANSS was also observed across rs12630592 genotypes in the context of \(FXRI\) rs496250 GG individuals. More specifically, in the context of \(FXRI\) rs496250 A-carrier genotype, subjects with rs12630592 TT genotype have higher \(\Delta\)-N-PANSS compared with \(FXRI\) rs496250 GG/rs12630592 GG (p= 0.01), \(FXRI\) rs496250 GG/rs12630592 GT (p= 0.007), and with \(FXRI\) rs496250 GG/rs12630592 TT (p= 0.03) individuals. Separate analyses on sample 1 and 2 indicated consistent results with those obtained on pooled Sample 1 and Sample 2 (see Supplementary Material).

Similar analyses on the Replication Sample found no main effect of \(FXRI\) rs496250 and \(GSK3\beta\) rs12630592 genotype, nor any interaction between the two genotypes, on \(\Delta\)-N-
PANSS. We reasoned that a possible interpretation of such an inconsistency may be related to the T0-T1 time interval used in the replication cohort. On this basis, we explored mean values of ∆-N-PANSS as a function of the different genotypic configurations. This inspection revealed that, in the context of FXR1-A-carrier genotype, GSK3β rs12630592 TT individuals had greater mean values of ∆-N-PANSS compared to GSK3β rs12630592 GG and GT subjects, which is consistent with directionality of results in the discovery sample.

No main effect of FXR1 rs496250 and GSK3β rs12630592 genotypes, nor rs496250-by-rs12630592 interaction was observed on the variation of the PANSS “Positive” and “General” subscale scores (all p-values >0.05).

3.3. Fxr1 gene expression is affected by AP treatment in the mouse brain.

ANOVA revealed PFC fxr1 expression was reduced (0.282 ± .077 fold, n= 4 animals per group) in mice treated with olanzapine as compared to those treated with vehicle (ANOVA: F (3.2), p<0.05).
Discussion

Previous evidence has suggested that the molecular interplay between FXR1 and GSK3β may have a role in the pathophysiology of SCZ, in particular in regulation of emotional phenotypes associated with NS of the disorder (12, 56). Previous findings have also implicated GSK3β in the mechanism of action of APs (53). Here we show that GSK3β interacts with FXR1 functional variation in modulating SCZ NS severity and response to APs and that, similarly to GSK3β (17-19), FXR1 expression is affected by APs.

In humans, we found that rs496250 and rs12630592 interact on NS severity and response to APs in patients with SCZ, providing evidence that the FXR1-GSK3β pathway is involved in modulation of such a symptom domain. In fact, we found that subjects carrying a genotypic condition associated with lower PFC GSK3β expression (rs12630592 TT individuals) show lower NS severity and greater response to APs in terms of negative symptomatology compared with other rs12630592 genotypes in the context of higher predicted FXR1 PFC expression (rs496250 A-carrier individuals). Notably, in previous work by our group (46) the same genotypic configuration (FXR1 rs496250 A carriers-GSK3β rs12630592 TT individuals) was associated with higher emotional stability and left amygdala activity during emotion processing. Therefore, it is possible that genetic regulation of brain FXR1-GSK3β expression, while modulating emotion processing and stability in healthy individuals, impacts on NS regulation in patients with SCZ. Such a possibility is in line with evidence that NS are associated with both emotional stability (48) and amygdala activity (36) in patients with SCZ.

Indeed, results on FXR1-by-GSK3β interaction on response to APs were not replicated in the current study, even though, when exploring mean values of delta PANSS as a function of the different FXR1-by-GSK3β genotypic configurations, we observed that in the context of FXR1-A-carrier genotype, GSK3β rs12630592 TT individuals had greater mean values of delta PANSS compared to GSK3β rs12630592 GG and GT subjects, which is
consistent with directionality of results in the discovery sample. Lack of replication in the
results might be due to the T0-T1 time interval used in the replication cohort, which is
designed to investigate quite fast responses to treatment and is shorter compared to those of
the discovery sample. This aspect may have prevented statistically significant detection of
subtle genetic effects on response. Furthermore, we found that chronic administration of
Olanzapine at a clinically relevant dose for 30 days to a sample of C57BL/6J mice was associated
with lower FXR1 transcription levels in mouse post mortem PFC compared with vehicle-treated
animals. This suggests that FXR1 is engaged in the chain of molecular events involved in the
mechanism of action of APs, or at least of Olanzapine, as previously reported for GSK3β. Quite
interestingly, a Transcriptome-Wide Association Study (57) has reported brain FXR1 mRNA
expression is up-regulated in patients with SCZ, a finding that might be in line with our current
result of an FXR1 mRNA reduction following chronic administration of an AP agent in mouse
brain. To our knowledge, this is the first study reporting a possible link between AP treatment
administration and FXR1 expression in brain. **We are aware that the animal model we here
adopted is limited by the exclusive use of olanzapine as AP treatment. Nonetheless, it
represents a strong indication for future replicative studies on animal models adopting
different AP drugs.**

At the protein level, GSK3β and FXR1 are involved in a negative regulatory interaction in
which GSK3β inactivates FXR1 (12). Therefore, it is possible that genetic factors modulating
brain expression of the two proteins, such as rs1263590 and rs4962590 allelic variation, may
amplify FXR1/GSK3β physiological signaling. Importantly, this signaling has been implicated in
a number of molecular events that may play a role in the regulation of NS severity and response to
treatments. For example, FXR1-GSK3β interplay has been implicated in dopamine signaling
mediated by D2 type dopamine receptor (25), that has been consistently linked with NS
pathophysiology and response to APs (12, 58-60). On this basis, it is possible that genetically
determined imbalance between GSK3β and FXR1 brain expression modulates the impact of
DRD2-mediated dopamine signaling on NS and their response to agents that primarily target and antagonize DRD2, i.e. APs. GSK3β activity is known to be regulated by DRD2 signaling and can be pharmacologically inhibited by APs having a DRD2 antagonist activity (17, 24, 61).

Furthermore, CRISPR/Cas9 mediated selective KO of GSK3β in DRD2 expressing neurons of the adult mouse PFC results in a reduction in social interaction, a prototypical proxy of human social withdrawal, which, in turn, is a typical NS of SCZ (62).

Another possible interpretation of our results is related to the relationship between GSK3β/FXR1 pathway, glutamate signaling, and phenotypes of relevance for NS of schizophrenia. In this regard, a recent report indicates that, in mouse, Fxr1 and Gsk3β modulate glutamatergic neurotransmission via regulation of AMPA receptor subunits GluA1 and GluA2, as well as vesicular glutamate transporter VGlut1 (56). Furthermore, other findings reveal that knockout of glutamate AMPA receptor 1 in mice (GluA1-KO) is associated with impaired social behavior (63), a prototypical model of SCZ NS (64, 65). Moreover, in humans, genetics and neurophysiology studies have consistently implicated glutamatergic signaling in NS (40, 66), and pharmacology investigation have suggested that such a signaling represents a potential target for NS treatment (67, 68). Furthermore, GRIA2 gene, coding for the glutamate ionotropic receptor AMPA type subunit 2 is associated with improvements in NS in patients treated with APs (69). It is thus possible that genetically determined modulation of the FXR1-GSK3β pathway in brain impacts on NS severity and response to APs by its intermediate tuning on glutamate neuronal signaling. Rather importantly, our results indicate that the impact of genetic variation potentially regulating FXR1-GSK3β signaling on psychopathology of Schizophrenia is quite specific for NS, while it is not significant for positive symptom domains are supported by different neurobiological and brain circuitry systems (70, 71), it is possible that genetic variation impacting on one of the two domains has less pronounced effects on the other one. This seems particularly plausible in light of existing evidence that
genomic variation associated with SCZ at genome-wide level of significance clusters to

different biological ontologies that distinctly support either positive or negative symptoms

(46)

As a whole, our results suggest that, while individually implicated in the pathophysiology
of SCZ by GWAS and molecular biology studies, FXR1 and GSK3β are players of one single
molecular pathway with a potential role in modulation of NS severity and response to APs. This
could be relevant to future set up of new pharmacological tools to treat NS domain of SCZ. We
are aware that a major limitation of our study is that genetic variation of rs12630592 and
rs4962590 is only a proxy of GSK3β and FXR1 PFC mRNA expression respectively, thus not
providing any actual measure of these genes’ transcriptional levels in the whole brain.

Additionally, mRNA expression itself is a proxy of one gene translation into the corresponding
protein. Therefore, it is possible that the genetic interaction we here observed is not directly due to
the exclusive contribution of GSK3β and FXR1 proteins to regulation of NS, so that other
molecular pathways, possibly related with GSK3β/FXR1 signaling, may be hypothesized behind
these clinical manifestations. **Another possible limitation of our study is that samples we
studied had quite a large internal heterogeneity in terms of AP medication used (olanzapine,
risperidone, quetiapine, paliperidone, aripiprazole, clozapine and haloperidol). Also, part of
the sample in the Discovery Sample was taking antidepressant and mood stabilizer
medication at the time of their recruitment in the current study. Further studies with
reducing such heterogeneity in terms of pharmacological agents used are warranted.**

In conclusion, findings of both our animal and human experiments highlight an
involvement of the FXR1-GSK3β signaling pathway in the pathophysiology and possibly
pharmaco-response of NS, thus shedding light on the intricate molecular basis of these complex
clinical phenomena and on possible new pharmacological treatments.

Author Contributions
Antonio Rampino wrote paper draft. All Authors contributed to draft revision and finalization. Antonio Rampino, Matteo Jacoviello, Silvia Torretta and Barbara Gelao performed statistical analyses on human subjects data. Federica Veneziani, Aleksandra Marakhovskaya and Jean Martin Beaulieu performed all animal experiments and analyses. Jean Martin Beaulieu supervised all animal experiments and analyses. Antonio Rampino, Rita Masellis, Silvia Torretta and Ileana Andriola contributed to human subject recruitment. Leonardo Sportelli contributed to data analysis. Antonio Rampino, Jean Martin Beaulieu, Giulio Pergola, Alessandro Bertolino and Giuseppe Blasi supervised final results and the overall paper scientific quality. Massimo Gennarelli, Alessandra Minelli, Chiara Magri and Antonio Vita provided clinical and genotype data of the Replication Sample. Giuseppe Blasi substantially contributed to revise paper draft and coordinate the study.

Declaration of competing interest

The authors declare no conflict of interest.

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Data Availability Statement

The data that support the findings will be available in the Open Science Framework.
repository at https://osf.io/d5e9t/ following a 6 month embargo from the date of publication to allow for commercialisation of research findings.
References


Figure Legends

Figure 1. Interaction between FXR1 rs496250 and GSK3β rs12630592 genotypes on Negative Symptom severity in the Discovery Sample. Subjects carrying GSK3β rs12630592 GG genotype and FXR1 rs496250 A-carrier have higher N-PANSS compared with GSK3β rs12630592 GT/FXR1 rs496250 A-carrier and with GSK3β rs12630592 TT/FXR1 rs496250 A-carrier subjects.

Furthermore, GSK3β rs12630592 GT/FXR1 rs496250 A-carrier subjects have higher N-PANSS than GSK3β rs12630592 TT/FXR1 rs496250 A-carrier subjects. Bar graphs show mean ± SE. * indicate 0.01 < p-value < 0.05. ** indicate 0.001 < p-value < 0.01. See text for detailed statistics.

Figure 2. Interaction between FXR1 rs496250 and GSK3β rs12630592 genotypes on Negative Symptom severity in the Replication Sample. In the context of FXR1 rs496250 A-carrier genotype, subjects carrying rs12630592 GG genotype have higher PANSS NS scores compared with rs12630592 T-carrier subjects. Furthermore, s12630592 GG subjects have higher PANSS NS scores than GSK3β rs12630592 GG/FXR1 rs496250 GG subjects. Bar graphs show mean ± SE. * indicate 0.01 < p-value < 0.05. ** indicate 0.001 < p-value < 0.01. See text for detailed statistics.

Figure 3. Interaction between FXR1 rs496250 and GSK3β rs12630592 genotypes on Negative Symptom response to Antipsychotics in the Discovery Sample. Subjects carrying GSK3β rs12630592 TT genotype and FXR1 rs496250 A-carrier have higher Δ-N-PANSS compared with GSK3β rs12630592 GT/FXR1 rs496250 A-carrier and with GSK3β rs12630592 GG/FXR1 rs496250 A-carrier subjects. Bar graphs show mean ± SE. * indicate 0.01 < p-value < 0.05. ** indicate 0.001 < p-value < 0.01. See text for detailed statistics.
Table 1. Genetic data distribution of the **Discovery Sample** (Sample 1 and Sample 2) and of the **Replication Sample**

<table>
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<tr>
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<th></th>
<th>Samples used to study the effect of genotypes on Negative Symptoms response to Antipsychotics</th>
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