Psychiatric disorders and SLC6A4 gene variants: possible efects on alcohol dependence and alzheimer's disease

Marco Calabrò1 · Laura Mandelli2 · Concetta Crisafulli1 · Stefano Porcelli2 · Diego Albani3 · Antonis Politis4 ·George N. Papadimitriou4 · Marco Di Nicola5 · Luigi Janiri5 · Roberto Colombo5 · Giovanni Martinotti6 ·Antonello Bellomo7 · Eduard Vieta8 · Stefano Bonassi9 · Alessandra Frustaci10 · Giuseppe Ducci11 · Stefano Landi12 · Stefania Boccia13 · Alessandro Serretti2

Abstract

Serotoninergic system is one of the most important neurotransmission systems investigated in the field of psychiatry. Exten-sive evidence reveals how alterations of this system, and especially of the SLC6A4 gene, may be associated with psychiatric disorders. In this study we aimed to evaluate the pleiotropic nature of SLC6A4 alterations and their association with the overall risk of brain diseases rather than disorder-specific. SLC6A4 variants, namely 5HTTLPR, STin2, rs2066713, rs25531, rs4251417, rs6354 and rs7224199 were investigated in 4 independent cohorts of subjects with specific psychiatric disorders, including Alcohol dependence disorder (ALC), Alzheimer disease (ALZ), Schizophrenia (SCZ) and Bipolar disorder (BPD). Other variables (biochemical parameters and Psychiatric scales scores) were also tested for association. SLC6A4 polymorphisms are not associated with the risk of developing major psychiatric disorders (SCZ and BPD); however some signals were detected in ALC (HTTLPR p- d = 9.25×10^{-03}, pr = 7.24×10^{-03}; rs2066713 p-d = 6.35×10^{-08}; rs25531 p-d = 2.95×10^{-02}; rs4251417 pd = 2.46×10^{-03}), and ALZ (rs6354 p-r = 1.22×10^{-02}; rs7224199 p-d = 1.00×10^{-08}, pr = 2.65×10^{-02}) cohorts.

Some associations were also observed on exploratory analyses. Our findings did not reveal any major influence on SCZ and BPD development; On the other hand, some alteration of the SLC6A4 sequence were associated with an increased risk of ALC and ALZ disorders, suggesting common pathways. The results of this study should be carefully interpreted since it suffers of some inherent limitations (e.g. cohort size, slight ethnic heterogeneity). Further analyses may provide better detail on the molecular processes behind SLC6A4 alterations.

Introduction

The Serotoninergic system is one of the main neurotransmis-sion systems of the Central Nervous System (CNS) where primarily innervates forebrain and midbrain structures [1]. It plays a major role in a variety of biological functions, including memory, appetite, sleep, cognition [1] and it is able to modulate Dopamine (DA) and Norepinephrine (NE) neurotransmission systems [2]. Also, affective-related behavior, self-control and emotional stability appear to be strongly modulated by this system [3, 4]. Medical research, in particular the psychiatric field, much focused on molecular processes related to the propagation and signal transduction of serotonin (5 -Hydroxytryptamine or 5-HT). Indeed, the data collected in the recent years pre-sented substantial evidence of how alterations within the serotoninergic system may increase the liability of psychi-atric disorders [5–8]. Its role as modulator of drug response was also widely investigated [9–11].

Specific key genes were originally investigated, including the ones encoding for monoamine oxidases (MAOs), Trypto-phan hydroxylases (TPHs), Serotonin receptors (5HTRs) and the serotonin transporter (SLC6A4) [12 , 13]. The latter, in particular, is believed to be one of most important modula-tors of serotonergic neurotransmission. SLC6A4 is localized on q11.1-q12 region of chromosome 17 and it encodes for a 12 trans-membrane spanning protein belonging to the family of sodium-and-chloride-dependent solute carrier family 6— member 4.

The transporter is responsible for the selective reuptake of 5-HT at nerve terminals, thus regulating the con-centration of the neurotransmitter in the synaptic cleft and influencing the magnitude and duration of 5 -HT signaling [14–16]. It also has a balancing role in 5-HT levels since it stores back 5 -HT molecules in presynaptic neurons, making them readily available for further use [17].

Variations of this transporter may be correlated to a wide range of causes: alteration of its structural amminoacidic sequence, expression variations caused by defective promot-ers, altered methylation mechanics and other epigenetic fac-tors, enhancer/silencer variations and many other ones may all cause alterations from the wild type structure/expression levels. Further, both genetic and epigenetic processes may be susceptible to environmental influences which further increase the difculty of the interpretation of the results from human association analyses. Despite these issues, functional and expression variations of SLC6A4 were widely investigated for susceptibility to the risk of psychiatric dis-orders [18–20]. Interestingly, despite the presence of several inconsistent results in literature, sequence alterations within this gene were associated with the risk for more than one psychiatric disorder. The data indicates how SLC6A4, as a central hub of serotoninergic-related molecular processes, may play a pivotal role in the development of these patholo-gies, though several other variables may influence this role as well. In light of these observations, the aim of our study was to test whether a set of variants within SLC6A4 may increase susceptibility to multiple psychiatric disorders and the role of specific genotypes as modulating factor of symptomatol-ogy in a series of psychiatric disorders. Multiple loci were already investigated for associations with psychiatric disor-ders (with heterogeneous results). A total of seven polymor-phisms were ultimately chosen for investigation in this study.
Among them, an insertion-deletion polymorphism in the SLC6A4 promoter region (5 -HTTLPR; rs4795541) has been associated with traits such as anxiety [21], emotional-related traits and psychiatric disorders as a whole [14]. This polymorphism consists in a variable repetition of a 20–23 base pair (bp) sequence. The two most common variants found in general population are defined as Long (L) allele with 16-repeats and Short (S) allele with 14-repeats [21]. From experimental data it was clear that the S allele causes a decreased transcriptional activity of SLC6A4, thus reduc-ing the reuptake activity compared to the L form [21]. Some studies have also demonstrated that the presence of rs25531 G allele (a polymorphism localized in the same region of 5-HTTLPR) in combination with the 5-HTTLPR L allele (LG) results in expression levels similar to S- carriers [22].
Of note, there is some controversy in literature regarding the effect of SLC6A4 variation of expression induced by S and L alleles and disorders. Mainly, a decreased expression is related to an increased risk of psychiatric disorders [23], but several data also evidenced an opposite association [23–25].
An additional variant widely investigated is localized in SLC6A4 intron 2. It is named STin2 (variable number of tandem repeats or VNTR) and it consists in the repetition of a nearly identical 17 bp sequence. Previous genetic analy-ses evidenced two major alleles (STin2.10 and STin2.12) corresponding to 10-, or 12-repeat units, and a number of low frequency variants, with the nine-repeat units allele (STin2.9) being the most common [26]. This polymorphic VNTR region acts as a transcriptional regulator, with the 12 -repeat allele being associated with an increased SLC6A4 expression [26]. We also included in the study rs2066713, rs4251417, rs6354, rs7224199, a group of polymorphisms previously investigated by our group [9], which were chosen according to the criteria described below (see Methods sections) and to enhance the coverage of SLC6A4 gene.

Methods
Cohorts
For the present study, seven samples with Caucasian ances-try, previously recruited in the context of other studies, were analyzed. The subjects were grouped according to the psy-chiatric disorders and were separately tested for association with SLC6A4 polymorphisms.
Alcohol dependence disorder (ALC)

An Italian cohort (64 Subjects and 32 Healthy Controls) and a Greek cohort (105 Subjects and 117 Healthy Controls) were recruited for this group. The criteria for inclusion are described elsewhere [27]. Briefly, to be included in the study patients had to fulfill the DSM-IV (Diagnostic and Statisti-cal Manual of mental disorders) diagnostic criteria for alco-hol abuse/dependence—"primary alcoholism" and to give full voluntary consent to the study. Other inclusion criteria were: no recent alcohol intake, absence of serious physical illness, absence of other drug abuse, absence of DSM-IV axis I co-morbidity. Patients and their relatives were enrolled in two centres (1) the Drug and Alcohol Addiction Clinic of the Athens University Psychiatric Clinic at the Eginition Hospital in Athens, Greece. (2) the Policlinic Institute "A. Gemelli"—IRCCS, Università Cattolica del Sacro Cuore in Rome, Italy.

Alzheimer disease (ALZ)
An Italian cohort (59 Subjects and 30 Healthy Controls) and a Greek cohort (94 Subjects and 127 Healthy Controls) were recruited for this group. Details on these cohorts were already described in previous studies [28, 29]. Regarding the Greek cohort, subjects were assessed with of the mini-mental state examination (MMSE) [30], the cornell scale for depression in dementia (CSDD) [31], the neuropsychiatric inventory (NPI) [32], Alzheimer's disease consensus—activ-ity of daily living (ADCS-ADL) and clinical dementia rat-ing (CDR) scale [33]. Patients of the Italian cohort were recruited among the subjects recruited for the GIGAS_ LOAD project. All participants were assessed through the administration of the Cambridge Examination for Mental Disorders of Elderly Persons—Revised (CAMDEX-R) [34].

Bipolar disorder (BPD)
An Italian cohort (120 Subjects and 128 Healthy Controls) and a Spanish cohort (135 Subjects) were recruited for this group. For the Spanish cohort, BPD out-patients were enrolled in a naturalistic cohort study by the Hospital Clinic of Barcelona, in Barcelona, Spain. Inclusion criteria were a diagnosis of Bipolar Disorder (type I or II) according to DSM -IV-TR criteria and age of at least 18 years. Subjects were assessed through the semi-structured interviews for Axis I disorders according to DSM-IV-TR criteria (SCID I). Italian subjects were enrolled if they met DSM-IV criteria for a diagnosis of BD type I/II. Patients of the Italian cohort were recruited in two national psychiatric centers: (1) the Department of Biomedical and NeuroMotor Sciences, Bolo-gna University. (2) the Psychiatric Unit of the University of Foggia, Italy. Further details are described elsewhere [35].

Schizophrenia (SCZ)
An Italian cohort (209 Subjects and 105 Healthy Con-trols) of SCZ subjects was included in this study as the SCZ cohort. Subjects that met the criteria of schizophrenia according to DSM-IV manual were recruited (1) among psychiatric inpatients of "San Raffaele Villa dei Fiori" facility, Rome, Italy, (2) by the Mental Health Department of ASL Roma 1 and (3) by the Psychiatric Unit, Depart-ment of Psychiatry, University of Bologna, Italy. Exclusion criteria were the presence of severe medical conditions or moderate to severe dementia. Clinical and demographic characteristics of patients were assessed at the recruitment. Details on the cohorts were described elsewhere [35].

Healthy control
Healthy subjects were recruited as controls in all of the centers involved in the study (controls divided between the cohorts, with partial overlap). No significant di fferences in genetic distributions was observed within the five con-trol cohorts. All subjects were included in the study only if they did not present any neurologic/psychiatric disease history or abnormal physical examinations.

All subjects and controls signed written informed con-sent and all protocols were approved by the local ethical committees as described in previous papers on the same cohorts referenced above.

Selection of polymorphisms

The following criteria were applied to select polymor-phisms: (1) literature evidence of involvement with psy-chiatric disorders (2) reported prevalence of at least 5% for the variant allele among Caucasians (data from http:// hapmap.ncbi.nlm.nih.gov/); and (3) availability of a vali-dated assay in the laboratories. The list of genotyped poly-morphisms is shown in Table 1. For each polymorphism is reported the wild type and the alternative allele. Their positions on the gene are reported in Fig. 1.

Genomic DNA was purified with an automated work-station (Maxwell, Promega, Fitchburg, MA, USA) and checked for quality with spectrophotometry (Nan-oDrop, Thermo Scientific). Genotyping was performed using (1) restriction fragment length polymorphism, (2) allele-specific oligonucleotide and melting curve analy-sis on Applied BioSystems 7500 Real-Time PCR system (Thermo Fisher Scientific Inc., Waltham, MA, USA) and high-throughput genotyping with the MassARRAY System (Agena Bioscience Inc., Hamburg, Germany). Genotyping was performed according to the manufac-turer's standard protocols. Forward and reverse primers' sequences are available upon request.

Table 1 Polymorphisms under investigation

Polymorphism	rs2066713	rs25531	rs4251417	rs6354	rs7224199	STin2	HTTLPR	HTTL-PRRs25531
Wild type allele	G	A	C	T	G	2.12	L	L_A
Polymorphic allele	A	G	T	G	T	2.10, 2.9	S	L_G, S

Fig. 1 Polymorphisms under investigation
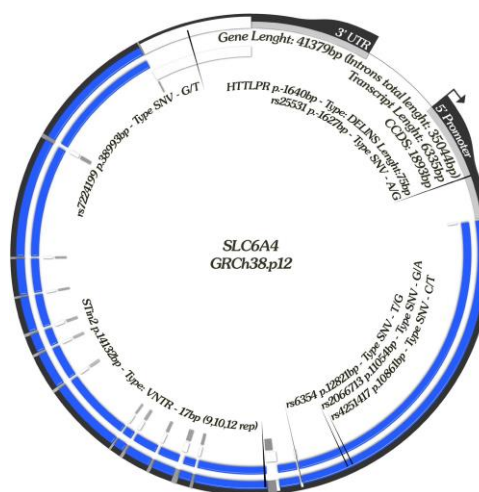
Outcomes under investigation

The main aim of the study was to test the associations between the investigated polymorphisms and the risk of developing a psychiatric disorder. Analyses were performed independently for each disease. Also, each subgroup was separately investigated as exploratory analysis in order to evaluate possible differences between populations. Finally, for each cohort further exploratory analyses were performed on the available biochemical parameters and psychiatric scales scores (for details please refer to Supplementary Table 1).

Statistical analysis

Haploview3.2 was used to test Hardy–Weinberg Equilibrium(HWE) [36]. The effect of markers was tested through logistic regression models in all groups to evalu-ate the risk of developing a psychiatric disorder. Dominant and Recessive models were tested in the cohorts and their

subgroups. Further tests were performed on the additional variables (Supplementary Table 1) where available. The combination of 5-HTTLPR and rs25531 was investigated by comparing LA/LA carriers versus other haplotypes as previ-ously suggested [37]. Covariates were selected according to their impact on outcomes. IBM SPSS package (http://www. ibm.com/analytics/us/en/technology/spss/) was used for the analyses.

In single marker analysis each group provided enough power (0.80) to detect risk alleles with odds ratios ≥ 1.89, ≥ 1.90, ≥ 1.79 and ≥ 1.90 for ALC, ALZ, BPD and SCZ respectively. The power was calculated through G*Power software v3.1.9.2.

To limit the probability of false positive finding we applied Bonferroni correction to our statistical tests. We took in con-sideration the number of SNPs tested for significance (7) and the number of datasets analyzed (7). Significance was consid-ered for $P < 0.05 = 0.00102$.

Results

Details of the cohorts under investigation are summarized in Supplementary Table 2. The genotypic distribution and results of HWE testing are reported in Supplementary Table 3.

Primary outcomes

According to our analyses, in all cohorts SLC6A4 polymor-phisms showed some influence in the risk of developing psy-chiatric disorders. The charts below report the effects (indi-cated as odd ratios) that SLC6A4 polymorphisms have on the risk. Both dominant and recessive model data have been reported. Details of these analyses and the results obtained from the diagnostic groups are reported in Supplementary Table 4 (Charts 1, 2).

Secondary outcomes

The exploratory outcomes tested for association showed some significant data. In particular, rs7224199 geno-type seems to be correlated with blood levels of Triglycerides in Greek Alcoholic subjects (Recessive model $p_r = 2.17 \times 10^{-03}$, Genotypic p-$g = 6.97 \times 10^{-03}$).

Greek Alzheimer subjects have shown variations of B12 levels related to rs4251417 genotype (Recessive model $p_r = 2.18 \times 10^{-03}$, Genotypic -$pg = 1.06 \times 10^{-02}$); of LPA levels related to rs7224199 genotype (Recessive model $p_r = 5.07 \times 10^{-05}$, Genotypic p-$g = 1.79 \times 10^{-04}$). The BPD cohort revealed associations between Hamil-ton depression rating scale (HDRS) scores and HTTLPR genotype (Dominant model -$pd = 1.03 \times 10^{-02}$, Genotypic $pg = 2.11 \times 10^{-03}$). In the same cohort young mania rating scale (YMRS) scores resulted modulated by rs7224199 genotype (Recessive model p-$r = 6.82 \times 10^{-04}$, Genotypic $pg = 4.11 \times 10^{-04}$). No other significant associations were found in the cohorts under analysis. Further details are reported in Supplementary Table 5.

Discussion

Overview of fndings. The serotonin transporter gene (SLC6A4) has been subject to relevant interest, since the central role of the coded protein in the serotonin neuronal activity [38], especially in relation to psychiatric disorders. According to our results, several of the polymorphisms investigated resulted having some modulatory effects. HTTLPR polymorphism was associated with the risk of developing Alcohol dependence in the cohort under analysis. In particular the significance of associations under both dominant and recessive models, indicated that only one S allele is needed to significantly increase the risk compared to a wild type genotype and two S allele further increase the possibility. Taking into consideration the combination with rs25531, only the recessive model maintained a significant association. Of note, in further exploratory analyses on the greek cohort after stratification in early and late onset subgroups this variant showed no significant associations (data not shown) . While the loss of significance may be attributed to the limited sample size (49 and 56 subjects respectively for early and late subgroups), it may also be related to the different genetic

architectures behind the two manifestations of Alcohol dependence. The expression of the 5-HTT gene is influenced by a 44 bp insertion/deletion polymorphism in its promoter region (5-HTTLPR) . In vitro studies have shown that the long (L) 5-HTTLPR variant led to a higher transcriptional activity compared to the short (S) variant [39]. It is also interesting to note that the presence of the S allele may impact on the development of cerebral structures, like amygdala, important in the emotion processing [40]. Overall this result suggests that a decreased concentra-tion of SLC6A4 gene products (S alleles reduces transcrip-tion levels of SLC6A4 mRNA) concur to increase the risk of Alcohol dependence liability, as supported by some literature evidence ([41] and references therein). It has to be noted, though, that some large meta analyses reported no association for this variants with Alcohol dependence dis-order [41], as such the data should be carefully evaluated.

Of note, according to our exploratory analyses, HTTLPR genotype can significantly influence depressive symptoms severity (HDRS scale) in the BPD cohort. S carriers showed the lower scores compared to L carriers; the lowest scores were evidenced in heterozygous subjects. Rs2066713 A allele has shown an increased risk for ALC (dominant model). No data was present in literature regard-ing the association of this SNPs and the this disorder, how-ever Yang et al. found rs2066713 being associated to another substance dependence-related disorder (nicotine) [42]. In silico sequence analyses through Human Splicing Finder software [43] revealed that the A allele breaks a potential consensus sequence for an Exonic Splicing Silencer (Motif 3—TCTCCCAA). Consequently, the A allele may alter the regulation mechanics of SLC6A4 mRNA maturation. However, no related experimental data was found in literature. Regarding rs4251417, we found some associations in the ALC group. In particular, the T allele was significantly more frequent on controls rather than ALC subjects. No cor-relations were found in literature between rs4251417 and the investigated disorders. However, the potential capacity of rs4251417 genotype to influence DNA methylation in SLC6A4 has been reported, thus impacting in the overall expression of the gene [44]. However, in silico analysis of the sequence with CpG island finder software (http://dbcat.cgm.ntu.edu.tw/) did not evidence any methylation focal point around the mutation. The in silico analysis for splicing alterations revealed that a G > A change of rs4251417 cause the creation of a branch point site. Also, the change causes the destruction of the consensus sequence for the Exonic Splicing Enhancer SRp40 protein. These alterations, though, do not seem to be sufcent to cause a significant impact on splicing. Of note, in the ALZ Greek subgroup, rs4251417 genotype was associated with vitamin B12 concentration variations in blood (C carriers showed lower values com-pared to TT carriers).

Recessive model association tests evidenced a correlation between rs6354 GG genotype and the risk of Alzheimer dis-ease. Though in silico analysis (CpG island finder) did not evidence any methylation focal point, literature data suggest a possible role of rs6354 in the regulation of DNA meth-ylation in SLC6A4 sequence, thus impacting in the overall expression of the gene [44]. In silico analysis on possible splicing alterations related to this polymorphism revealed that the T > G change causes the creation of a branch point site. Also, G carriers have a consensus sequence for an Exonic Splicing Silencer (Motif 3—TCTCCCAA). How-ever, given the localization of the polymorphism and the strength of these alterations, rs6354 genotype does not seem to cause a significant impact on splicing.

Rs7224199 T allele (dominant model) and TT genotype (recessive model) have a protective effect against ALZ. This trend (but not significant overall) could also be observed in the other disorders. According to exploratory analyses, rs7224199 may also influence the severity of manic symp-toms in BPD subjects, with TT carriers exhibiting higher scores compared to G carriers. Of note, Biochemical param-eters like Triglycerides and LPA in Greek ALC and ALZ subgroups, respectively were found different between sub-jects and controls. In silico analysis on possible splicing alterations related to this polymorphism revealed that the G > T change cause the loss of a splice site and of an Exonic Splicing Silencer consensus sequence. According to the software, it also introduces an Exonic Splicing Enhancer consensus sequence. However, these changes do not seem to cause a significant impact on splicing. Also, no role on methylation mechanic was proposed for this polymorphism.

No associations were observed for STin2 in the primary analyses. STin2 is localized in a enhancing region, whose activity/efciency is significantly correlated with the number of repeats within STin2. In particular, it was demonstrated that the higher the number of repeats, the more enhancing factors are recruited in this region. As such, the 2.12 allele translates in a higher expression activity of SLC6A4 [26]. Despite this, this change in expression levels does not seem to be enough to influence risk in our cohorts.

Limitations
The results of this study should be carefully interpreted since limited sample size, slight ethnic heterogeneity, and deviation from HWE of some SNPs which may have acted as confounding factors for the analyses. It has to be noted that deviation from HWE seems to be a common occurrence for SLC6A4 polymorphisms [9, 45]. We did not extensively investigate the genetic structure of SLC6A4, as such our data does not consider the presence/absence of other variants.

Conclusion
Variants within SLC6A4 sequence were found to be corre-lated mainly with ALC and ALZ disorders. We observed a correlation between HTTLPR S allele with an increased risk of developing an Alcohol dependence, in line with some lit-erature data. No correlations were found between HTTLPR variants and ALZ. This lack of association is also supported in literature [46]. About the other variants, rs2066713 was associated with the risk of Alcohol dependence and rs6354 with Alzheimer. Also, rs7224199 variations seemed to play a protective role against Alzheimer. The central serotonergic functioning is correlated of with impulsivity and aggression [47], moreover, the level of 5-HT functioning may impact the behavioral effect of alcohol as observed in literature data [48]. According to the data, SLC6A4 may indeed play a role in the molecular processes correlated with ALC and ALZ, but, at least in our cohorts, this role cannot be extended to all psychiatric disorders. Indeed, despite the amount of support in literature, we failed to correlate SLC6A4 altera-tions with any of the major psychiatric disorders (BPD and SCZ). Although the limited sample size could explain the lack of associations, recent works described the possibility of a more limited role for this gene compared to what we were used to know by literature [49]. It is possible for this gene to be capable of influencing the risk of psychiatric disorders, but only in specific genetic subsets of the popu-lation. Thus, more specific criteria for recruitment should be taken in consideration, in order to avoid the heteroge-neity typical of large studies, as evidenced by Cai et al. [50]. In particular, with respect to alcohol use disorder, a stratification in early and late onset subgroups may better define the role of SLC6A4 variants, since the significance may vary among the two groups [51]. Further analyses may help to better detail the molecular processes behind SLC6A4 variants, because it is not enough to explain the complex molecular picture behind these disorders. As such, further interactions with other key element should be considered for future investigations.

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Affiliations

Marco Calabrò1 · Laura Mandelli2 · Concetta Crisafulli1 · Stefano Porcelli2 · Diego Albani3 · Antonis Politis4 · George N. Papadimitriou4 · Marco Di Nicola5 · Luigi Janiri5 · Roberto Colombo5 · Giovanni Martinotti6 · Antonello Bellomo7 · Eduard Vieta8 · Stefano Bonassi9 · Alessandra Frustaci10 · Giuseppe Ducci11 · Stefano Landi12 · Stefania Boccia13 · Alessandro Serretti2

1   Department of Biomedical and Dental Sciences and Morphofunctional Imaging, University of Messina, Messina, Italy

2   Department of Biomedical and NeuroMotor Sciences, University of Bologna, Viale Carlo Pepoli 5, 40123 Bologna, Italy

3   Laboratory of Biology of Neurodegenerative Disorders, Neuroscience Department, Istituto di Ricerche Farmacologiche Mario Negri IRCSS, Milan, Italy

4   Department of Psychiatry, University of Athens Medical School, Eginition Hospital, Athens, Greece

5   Fondazione Policlinico Universitario "A. Gemelli" - IRCCS, Università Cattolica del Sacro Cuore, Rome, Italy

6   Department of Neuroscience, Imaging and Clinical Sciences, University "G. d'Annunzio" of Chieti, Chieti, Italy

7 Psychiatry Unit, Department of Medical Sciences, University of Foggia, Foggia, Italy

Chart 1 Dominant model. Association results on the risk. In the above diagram are showed the results of asso-ciation tests under a dominant model construct. In this model the presence of at least one mutated (-) allele was tested against the homozygous wild-type genotype (wt/wt). The Odds Ratios (plus Confidence Intervals) are reported on the X axis in a logarithmic scale. Data on the right of Y axis indicates causative effects toward the risk. Data on the left indicates pro-tective effects. An * indicates the significant results from our analyses. The pValues are here summarized: **ALC** (HTTLPR pd = 9.25×10^{-03}; rs2066713 pd = 6.35×10^{-08}; rs25531 pd = 2.95×10^{-02}; rs4251417 pd = 2.46×10^{-03}) and **ALZ** (rs7224199 pd = 1.00×10^{-08}). For more details please refer to Supplementary Table 4
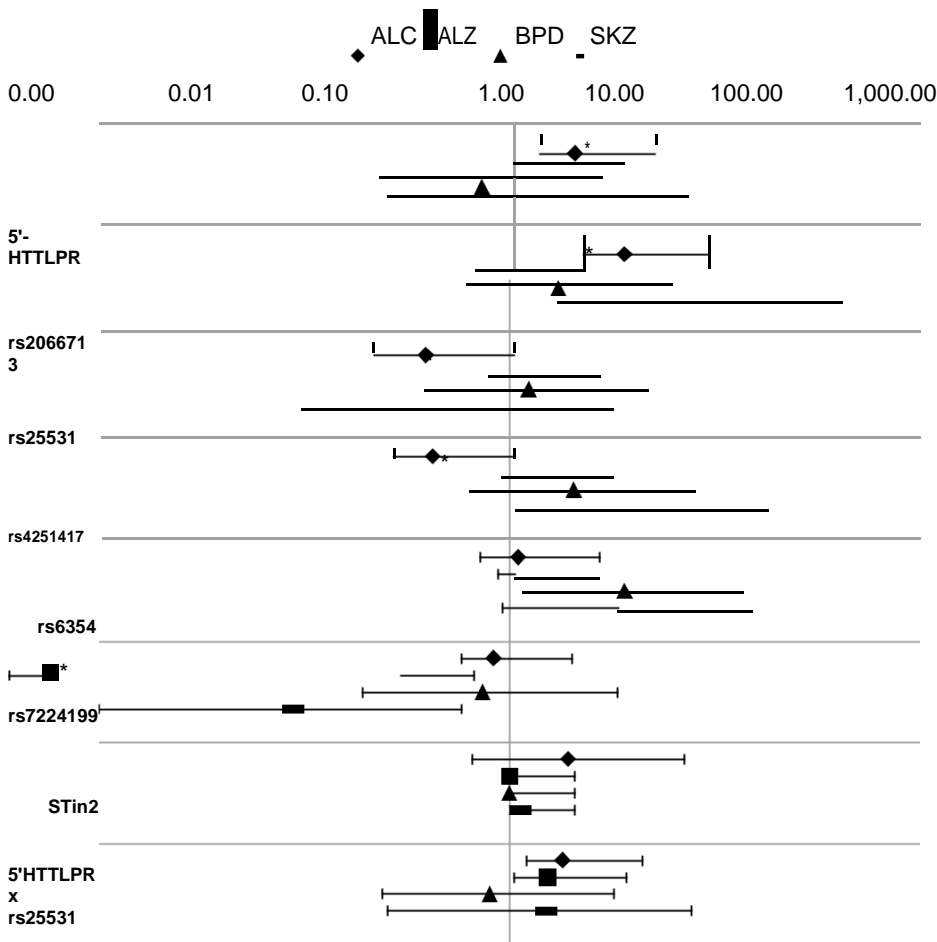
Chart 2 Recessive model. Association results on the risk. In the above diagram are showed the results of asso-ciation tests under a reces-sive model construct. The
-/- genotype was tested against wt/- and wt/wt genotypes. The Odds Ratios (plus Confidence Intervals) are reported on the X axis in a logarithmic scale. Data on the right of Y axis indicates causative effects toward the risk. Data on the left indi-cates protective effects. An * indicates the significant results from our analyses. The pValues
are here summarized: **ALC** (HTTLPR pr = 7.24×10^{-03};
HTTLPR x rs25531
pr = 7.85×10^{-03}) and **ALZ** (rs6354 pr = 1.22×10^{-02}; rs7224199 pr = 2.65×10^{-02}).
For more details please refer to Supplementary Table 4