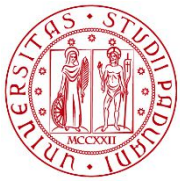

UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari

CORSO DI DOTTORATO DI RICERCA IN: SCIENZE MEDICHE CLINICHE E SPERIMENTALI

CURRICOLO: NEUROSCIENZE

CICLO 29°

5HTTLPR POLYMORPHISM, STRESSFUL EVENTS, NEUROPSYCHOLOGICAL PERFORMANCE AND BRAIN CONNECTIVITY IN EATING DISORDERS

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Abstract

Introduction. Low functioning variants of 5HTTLPR have been associated to an increased risk of depression in subjects who experienced stressful events, to altered cognitive functioning and decisional processes, and functional and structural neural patterns. Contrasting evidence is available up to now in Eating Disorders (ED), and no study has evaluated the polymorphism effect on brain connectivity according to graph theory in Anorexia Nervosa (AN).

Methods. We recruited up to 735 patients with life-time history of AN or bulimia nervosa (BN) according to DSM-IV criteria and up to 241 healthy controls (HC) for the assessment of the association between 5HTTLPR polymorphism and ED. We merged our Biobank data from BIO.Ve.D.A. and meta-analyzed 22 former studies. Patients underwent a structured diagnostic interview for present or life-time ED, an interview for presence and severity of stressful events, Edinburgh Handedness Inventory, Wisconsin Card Sorting Test, Trail A making test, Trail B making test, Iowa Gambling Task, Cognitive Bias Task, psychopathology rating scales for ED and general symptoms. Finally patients with AN and HCs underwent a Magnetic Resonance; their brains' connectivity integration and segregation measures were then measured with Graph Analysis Toolbox, according to 5HTTLPR polymorpshim.

Results. Our results from a meta-analysis including data from BIO.Ve.D.A. and 22 previous studies, suggest that 5HTTLPR polymorphism does not have a role per se in determing ED onset. However it may moderate the effect of SEs in increasing the risk of ED onset, and the influence of SEs on ED severity, anxious, depressive and obsessive symptoms. When we tested both a multiplicative and an additive model, which is considered to be more representative of a real-world gene by environment interaction, such a 5HTTLPR by SE interaction was not confirmed instead. S allele was associated with worse performance at Cognitive Bias Task and Trail Making B, and with increased ED psychopathology, general psychopathology, anxious, depressive, and obsessive symptoms. Finally S allele was associated with decreased segregation measures at brain connectivity analysis according to graph theory compared with L allele in AN; this was an opposite association compared with healthy controls who had higher modularity associated with S allele instead.

Conclusions. 5HTTLPR polymorphism does not seem to be a causal factor of ED per se, but it seems to play a role in moderating the role of stressful events in increasing ED risk. Such a moderation however did not reflect a gene by environment interaction according to either a multiplicative or additive model. S allele was associated with higher psychopathology scores, and worse neuropsychological functions in AN, and with a disrupted segregation measures of brain signal connectivity compared to HCs.

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1. 5HTTLPR POLYMORPHISM IN PSYCHIATRY

Psychiatric disorders pathogenesis is well represented by a multifactorial model, including genetic, and environmental factors. As regards genetics, increasing body of evidence has focused on 5HTTLPR polymorphism. 5HTTLPR is a 5' regulatory promoter region on chromosome 17 that has been modelled as being bi-allelic in nature depending on a 44bp deletion or insertion, with the S allele being low functioning, and the L allele being high functioning (fig. 1) (1). Moreover, a tri-allelic model has been illustrated, with the Lg (an L allele with Gre placing A in its SNP sequence) allele functioning equivalent to the S allele(2, 3). This polymorphism has been described having different distributions in different ethnicities (4), has been studied in several different psychiatric disorders, and in general population. However contrasting results emerged, which we now focus on, with this brief introduction about the role of 5HTTLPR in psychiatry.

Several historically grounded reasons and more actual scientific evidence support the potential clinical impact of 5HTTLPR polymorphism. The monoaminergic theory of depression which originated from clinical observations in 1950s and which has then been supported by several pieces of both clinical and pre-clinical evidence(5), suggests a central role of serotonin in pathogenesis of major depressive disorder (MDD) (6) together with other monoamines (7). Thus several authors have focused on the possible role of 5HTTLPR polymorphism in MDD. 5HTTLPR short allele homozygosity has been associated with increased risk of depression(8) compared to long allele homozygosity in females, and the same results have been confirmed in a recent meta-analysis (9) including 3392 cases and 5093 controls from 23 studies (S allele homozygosity; OR=1.33, 95%CI 1.19-1.48 - S allele presence; OR=1.16, 95%CI 1.08-1.23). Also, S allele has been associated with earlier MDD onset in a Japanese population(10), and notably long allele has been associated with faster response to sertraline in elderly patients affected by MDD(11), and better response to psychotherapy in children with anxiety disorders(12). The same recent meta-analysis mentioned above has also associated low functioning 5HTTLPR polymorphism with an increased risk of alcohol dependence(9) (S allele homozygosity; OR=1.18, 95%CI 1.01-1.38 - S allele presence; OR=1.26, 95%CI 1.01-1.23). Furtherly widening the spectrum of psychiatric conditions whose risk could be increased by being an S-allele carrier, also antisocial and borderline personality disorders have been reported to be more frequent in S allele carriers in low-income countries(13). In patients with Bulimia Nervosa (BN) symptoms short allele has been associated with novelty(14) and sensation seeking, insecure attachment(15) and dissocial behavior(16) in presence of life-time physical or sexual abuse. Also, within patients with BN, harm avoidance has been reported higher in those patients with S allele compared to those without it(17). Short allele genotype has also been associated with antisocial behaviors(18) in a meta-analysis of studies including human samples, and with aggressive behaviors

in children as well(19). Moreover, property offending behaviors under the effects of cannabinoids has been described in African American females who carried 5HTTLPR short allele(20). Finally, higher harm avoidance, alongside with higher scores at Beck Depression Inventory have been described in young subjects with excessive internet use(21). Beyond diagnostic entities, 5HTTLPR polymorphism has also been associated with dimensional personality and temperament characteristics in general population. In a community sample S allele has shown higher avoidant personality scores at International Personality Disorder Examination(22). Moreover, in a sample of 169 healthy females with no psychiatric condition, anxiety, depression, hopelessness, guilt, hostility, aggression, presence of neurotic symptoms, self-directedness scores, and affective temperaments carrying a depressive component have been associated with S allele, even after controlling for age(23). Finally, in 139 Caucasian psychiatrically healthy women, hyperthymic temperament as measured by TEMPS-A, the least associated with affective disorders(24), was the only temperament domain which was not associated with S allele(25).

However despite such a body of evidence suggesting a role of low functioning 5HTTLPR in severe psychiatric disorders, several negative results have not confirmed a role of this polymorphism in mental illness. For example, in Japanese population the association between S allele and increased depression risk has not been confirmed(10). Beyond depression, considering the wider affective disorders spectrum, no association has been described in a large European multicenter case-control study involving 539 unipolar affective disorders, 572 bipolar patients, and 821 control(26). Additionally, neither a mediator role on response to psychotherapy has been replicated for 5HTTLPR, even if negative results were limited to childhood anxiety disorders(27). Furthermore, beyond depression and affective disorders, in a meta-analysis we published in World Journal of Biological Psychiatry in 2016 and that we are presenting in the following chapter of the present final dissertation, when we accounted for ethnicity related heterogeneity, and for publication bias, neither Eating Disorders (ED) were associated with the short allele of the promoter of the gene for the serotonin transporter(28).

Based on such a body of conflicting evidence, we have investigated the role of 5HTTLPR in ED as a risk factors per se, its interaction with stressful events according to a gene by environment model, its effects on neuropsychological functions within ED, and the possible neural pathways underpinning such functions in a sample of patients with AN.

1. 5HTTLPR POLYMORPHISM IN EATING DISORDERS

(Original manuscript has been published in World J Biol Psychiatry. 2016 Jun;17(4):244-57)

2.1 INTRODUCTION

5-HTTLPR polymorphism is by far the most studied polymorphism in psychiatry, and growing interest is focusing on its role in eating disorders (ED) as well. 5HTTLPR is a 5' regulatory promoter region on chromosome 17 that has been modelled as being bi-allelic in nature depending on a 44bp deletion or insertion, with the S allele being low functioning, and the L allele being high functioning (fig. 1) (1). Moreover, a tri-allelic model has been proposed,with the Lg (an L allele with Gre placing A in its SNP sequence) functioning equivalent to the S allele. Low functioning variants of this triallelic polymorphims have been associated to an increased risk of depression in subjects who experienced stressful events(8), to altered cognitive functioning(29) and decisional process, and to precise functional and structural neural patterns. Despite some former meta-analyses suggesting an association between the short/low functioning 5HTTLPR allele (S) and anorexia nervosa (AN), contrasting evidence is available up to now. Several studies have investigated the relationship between AN, BN and ED and 5HTTLPR. However, only one focused on binge ED, and only two compared patients affected by AN with an obese sample. These studies have partially been meta-analysed with literature searches conducted up to July 2008 (30) and October 2009(31). However, more individual studies have explored this area since then, after 2009 (last search date from previosu authors).

The aim of this part of the study is to meta-analyze studies that investigated the association between 5HTTLPR polymorphism and ED, merging available data in literature and data from the 'Biobanca Veneta per i Disturbi Alimentari' (BIO.VE.D.A.)

2.2 METHODS

Participants, DNA sampling and analysis. The 'Biobanca Veneta per i Disturbi Alimentari' (BIO.VE.D.A.) sample included 735 patients with a lifetime history of AN and/or BN according to DSM-IV criteria, and 241 HC. Patients were recruited in five Eating Disorder Units of the Veneto region, Italy. The BIO.VE.D.A. project is funded by the Veneto Region and aims to create a genetic biobank for ED (30). Inclusion criteria were a life-time diagnosis of AN or BN according to DSM-IV (32), age >14 years old, patients' and parental (if less than 18 years old) informed consent. The study was approved by the local hospital Ethic Committee. Exclusion criteria were organic comorbidity or major psychiatric comorbidity (bipolar disorder, schizophrenia, major depressive

disorder). After informed consent, all participants underwent a saliva or blood sample. Genomic DNA was extracted from 200ml of whole peripheral blood, using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH), or from 500ml of saliva using an Oragene DNA/saliva Kit (DNA Genotek Inc.) according to the manufacturer's instructions. The 5-HTT promoter region was amplified from genomic DNA to discriminate between the L- and S-alleles (rs4795541). Primer sequences were designed using Primer 3 software (http://frodo.wi.mit.edu/) (forward: CAACTCCCTGTACCCCTCC T and reverse: GTGCAAGGAGAATGCTGGAG). This reaction produced a fragment of 297 bp for the long L allele and a fragment of 254 bp for the short S allele. A total of 200ng of genomic DNA was amplified using 12.5ml of prealiquoted ReddyMix PCR Master Mix (Thermo Fisher Scientific, Milan, Italy), 10 X DMSO and 0.4mcM of each primer, in a total volume of 25ml. Cycling conditions were: 1 cycle at 94°C for 4min, followed by 37 cycles at 94°C for 1min, at 61°C for 1min and at 72°C for 1min. To genotype the rs25531 the amplification was followed by a restriction digestion. A total of 7ml of PCR product was digested at 37°C for 2h with 5U of MspI (New England BioLabs, Celbio, Milan, Italy), which recognises the sequence 50-C/CGG-30. Fragments were separated on acrylamide gel at 10% (45min at 200V). The LA allele produces fragments of 257 and 39 bp, while the Lg allele produces fragments of 174, 84 and 39 bp.

Search strategy. We conducted an electronic literature search in PubMed from database inception until 1 April 2015 for studies investigating 5HTTLPR biallelic or trialleic genotype or alleles frequencies in ED, with or without a control group. Controlled vocabulary terms (MeSH) and the following keywords were used in the search strategy: ((''Anorexia Nervosa''[Mesh]) OR (''Bulimia Nervosa''[Mesh]) OR (''Eating Disorders''[Mesh]) OR (''Binge-Eating Disorder''[Mesh]) AND (serotonin transporter OR 5HTTLPR OR 5-HTTLPR)). Reference lists of included articles and those relevant to the topic were hand-searched for identification of additional potentially relevant articles.

Study selection. Included were only studies that: (1) included patients affected by ED according to DSM-IV criteria and (2) reported 5HTTLPR genotype or allele frequencies in patients affected by ED, and a control group, if present. Both biallelic and triallelic modelled studies were analysed. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) criteria (33) were used for the quality assessment of included studies. Previous reviews and meta-analyses were fulltext read to identify further studies.

Data extraction and statistical analysis. Two authors independently extracted data from the selected studies into a standardised Microsoft Excel spreadsheet. Any disagreement was resolved by consensus. The following information was extracted: (i) study population characteristics (e.g. sample size, demographics, diagnostic criteria and subtype of AN or BN, comorbidities, medications,

anthropometric data); (ii) biallelic or trialleic genotype or alleles frequencies in patients and controls; (iii) quality indicators used for the STROBE assessment; and (iv) Hardy–Weinberg equilibrium (HWE). Whenever studies had the same authors or affiliation or close publication year, we contacted authors to verify data were not referring to the same sample. Whenever genotype or allele frequencies were not available, we contacted authors to ask for unpublished data. Furthermore, we extracted data from a previous meta-analysis (34), reporting data not available in original papers. We ran the following comparisons within patients and within controls: (1) triallelic low-functioning genotype frequency (containing S or Lg) vs. high-functioning genotype (LaLa); (2) biallelic low-functioning genotype (SS or SL) vs. high-functioning (LL); (3) triallelic low-functioning allele frequency (S and Lg) vs. high-functioning allele frequency (La); (4) biallelic low-functioning allele frequency (S) vs. high-functioning allele frequency (L). Additionally, comparing ED, AN and BN patients with the respective healthy control group, we ran the following comparisons: (5) biallelic low-functioning genotype frequencies (SS or SL), including recessive model testing for the low-functioning allele; (6) biallelic low-functioning allele frequencies (S); (7) triallelic low-functioning genotype (containing S or Lg); and (8) triallelic lowfunctioning allelic frequencies (S and Lg). When an obese sample was used as a control group(7, 9), we compared it separately to the ED samples. Moreover, we tested also dominant models for the low-functioning allele in ED, AN and BN vs. HC. Finally we compared the bi-allelic model vs. triallelic model, reporting differences in frequencies from studies providing both bi-allelic and triallelic data. Grouping of SS and SL was based on the presumed dominant role of the S allele of 5HTTLPR polymorphism. We meta-analysed comparisons reported in at least two studies, in order to provide the maximum number of meta-analyzable outcomes. The meta-analysis was performed using Review Manager(35) Version 5.1 for Windows. When combining studies, the random effects model (36) was used to account for study heterogeneity. For dichotomous data, odds ratio (OR) with its 95% confidence interval (CI) was used. Study heterogeneity was measured using the chi-squared and I-squared statistics, with chi-squared $p>0.05$ and I-squared>50% indicating significant heterogeneity (37). If I-squared>50% a sensitivity test was performed, where each study, one at a time, was excluded from the overall OR calculation to examine if any single study contributed significantly to the overall result. Furthermore, since there is evidence of different distributions of 5HTTLPR in specific ethnicities, e.g. higher S allele frequencies in Japanese populations compared to Caucasian populations, we ran a sensitivity analysis with studies including other than Caucasian populations. Finally, funnel plots were inspected visually to assess the possibility of a publication bias. We included Bio.Ve.D.A. group data in the meta-analysis. HWE was tested in each study separately with both chi-squared test and relative excess heterozygosity test (REH) (38). Finally, we

tested the pooled REH from all included studies using a random effect model, which is a more appropriate tool than the chi-squared test to test HWE in meta-analyses(38).

2.3 RESULTS

Results from this part of the project have been published on World Journal of Biological Psychiatry(28).

BIO.VE.D.A. Data. The BIO.VE.D.A. sample includes 735 patients affected by ED and 241 HC. Among ED, 526 were affected by AN lifetime, 341 by BN lifetime. Demographic features, genotype and allele frequencies are displayed in Table 4. No differences were found in both biand tri-allelic allele frequencies, or in genotype frequencies, between ED, AN, BN and HCs, or between different EDs.

Hardy–Weinberg equilibrium and relative excess heterozygosity test. All included studies were consistent with HWE and REH (38) according to random effects model; only one study(39) reported data from a sample with a lower-than-expected l/l frequency.

Included studies. We included BIO.VE.D.A. data and data from 21 studies, according to the flow chart shown in Figure 2. We excluded one study because it allowed for subclinical participants; however, this study was included in a previous meta-analysis(31). The main features of the 22 included studies are summarised in Table 1. Altogether, 16 studies compared patients affected by ED with non-affected controls; six studies did not include a control group. Of the 16 control groups, 12 studies used HCs, two included obese subjects, one underweight subjects one normal eaters, one healthy sisters (40), and one parents (41). All studies defined ED according to DSM-IV criteria. Sixteen of the 22 studies used a biallelic model, six also used a triallelic. The quality of the included studies is available upon request.

Analyses within patients and within controls. Results of the meta-analyses within patients and within controls are reported in Table 2. Within patients and within controls, low-functioning genotypes were more frequent than high-functioning ones (ED vs. controls, $P<0.0001$). Within patients and within controls, high-functioning alleles were more frequent than low-functioning ones in the biallelic model. This result held true even in a leaveone-out sensitivity analysis, which based on funnel plot inspection excluded Matsushita et al. from the analysis. However, adopting the triallelic model, no significant difference emerged between low- and highfunctioning allele frequencies. Comparing the two models, only including studies using both the biallelic compared with the triallelic model included 9.9% more low-functioning genotype and 13.7% more low-functioning alleles.

Analyses comparing patients vs. controls. All results of the meta-analyses comparing patients with control groups are reported in Table 3. Low-functioning genotype frequencies in patients groups, with both biand tri-allelic models, did not differ from control groups (Figure 3). Furthermore, in both bi- and triallelic models, low-functioning allele frequencies in ED and in BN patients (biallelic only), were not different from control groups. Low-functioning allele frequencies in AN, in the Biallelic model, were significantly higher than in control groups (P=0.03). However, we removed one clearly outlying study (42) from the analysis based on funnel plot inspection, and as the patient group was more than 3 times smaller than the control group; the result became non-significant (P=0.09) (Figure 4). Finally, we did not find that a dominant model significantly explained the group difference between AN (P=0.13, after removing Matsushita et al, BN (P=0.85), or ED (P=0.37), vs. HC.

ED patients vs. obese patients. Only two studies compared patients affected by ED with patients affected by obesity. Low-functioning genotype and allele frequencies were not significantly different between the two groups (genotype P=0.37; allele P=0.40).

2.4 DISCUSSION

Several lines of evidence show that an abnormal functional activity of the 5-HT system might affect satiety, anxiety and mood disorders. However, according to our data, 5HTTLPR polymorphism did not appear to have any direct additive effect on the risk of developing ED. Thus, although two previous meta-analyses(30, 31) reported significant associations between the low-functioning allele of 5-HTTLPR polymorphism and the risk of having AN, the newly accumulated evidence shows contrasting evidence about the role of this polymorphism in ED – including AN. Similarly to what has been observed in other psychiatric disorders (41-44), an additive effect of a single polymorphisms is not a valid model to explain the pathogenesis of ED. Therefore, studies exploring interactive effects are needed. In the present study, a comparison between patients vs. controls was performed to re-test previously investigated associations with updated evidence. Both in the BIO.VE.D.A. data and in our meta-analysis, we found no significant difference in low-functioning genotype and allele frequencies between ED and controls. Regarding BN, a previous meta-analysis (14) pointed out that the relationship between the serotonin transporter gene polymorphism variance and BN was still controversial. In our meta-analysis, we added not only one more study to the pooled data, but we added the BIO.VE.D.A. data as well, including evidence from the largest sample published up to now. Our data confirm, like the previous findings (30), the absence of any difference in the 5HTTLPR polymorphism distribution in ED vs. controls. This finding seems to rule out serotonin transporter polymorphism as a causal factor per se in the ED pathogenesis. Another unique contribution of this meta-analysis is the comparison of allele and genotype frequencies between patients with ED and

individuals affected by obesity. This comparison also showed no difference between the two groups, suggesting no relationship between BMI and 5HTTLPR polymorphism, as previously reported in a psychiatric sample. However, we acknowledge that a meta-analysis including only two studies is very limited, so more studies are needed in this area. Furthermore, more studies should investigate the link between serotonin transporter polymorphism and BMI, in both healthy and psychiatrically ill subjects. We also tried to investigate samples affected by BED in our analysis, but to our knowledge only one study (43) has investigated serotonin transporter polymorphism in that population, precluding any meta-analytic assessment. The second part of our analysis was to evaluate the impact of the triallelic model in comparison to the biallelic model. Since the biallelic model classifies Lg alleles as high-functioning, which are actually lowfunctioning, a triallelic model is preferred since it better describes the ‘‘functional’’ distribution of the different alleles and genotypes. To date, only three studies in six studies used a triallelic model. Finally, our analyses within patients and within controls add to the available knowledge about the epidemiological distribution of the 5HTTLPR genotype, showing that low-functioning genotypes were significantly more frequent than high-functioning genotypes in both ED and controls. This finding could be due to the fact that low-functioning genotypes include two out of three (SS+SL vs. LL in biallelic model) and five out of six (SS+SLa+SLg+LgLg+LgLa vs. LaLa in triallelic model) possible combinations, respectively. However, other reasons could also play a role, including pleiotropic effects of the 5HTTLPR polymorphism and unknown advantages of a low-functioning 5HTTLPR genotype under certain conditions. More research in this area is needed to further clarify this finding and its potential implications. The present study has both strengths and limitations that need to be taken into consideration when interpreting its results. First, the BIO.VE.D.A. biobank provides the largest sample of patients affected by ED (compared to a healthy control group) that has been investigated regarding the polymorphism of the serotonin transporter gene. Furthermore, BIO.VE.D.A. data agree with the results of the meta-analysis (considering the sensitivity analysis invalidating the association of the S allele and S or SL genotype with ED compared to controls). Moreover the BIO.VE.D.A. sample is free from major psychiatric comorbidities (bipolar disorder, schizophrenia, major depressive disorder), removing the possibility of potential biases due to other major psychiatric disorders. Table 1 presents data showing how previous studies often did not control for depressive disorders that are frequently encountered in patients with ED. Thus, authors firmly promote multi-centric collaborations involving national health services both within and among countries. Second, with seven additional studies published in the last 6 years since the last meta-analyses, this meta-analysis increased the sample size from 2,105 to 3,736 patients and from 2,032 to 2,707 controls. Third with our search we also explored the relationship with BED and obesity, pointing out the need

for more studies in order to build some reliable evidence on the relationship between 5HTTLPR, BED, and obesity. Fourth, we stressed the need for more Asian studies, since compared to Caucasians the S allele seems more frequent in Asian people, in particular in the Japanese(44, 45). Fifth, compared to the meta-analysis by Lee and Lin(30), we increased the sample size of BN from 395 to 824 patients, without mixing in sub-clinical samples, as others (31) did. While we agree that a dimensional investigation is necessary to explain the relationship between symptoms and genes as proposed by Calati(31), we suggest that specifically designed studies are needed to provide consistent evidence on this dimensional aspect of ED. Conversely, mixing in minimally symptomatic subjects with ES is not helpful, adding more noise than clarifying dimensionality aspects. Moreover, our paper differs from Calati, in that we only metaanalysed studies with comparisons between patients and control groups, instead of building a virtual control group for studies without concurrently collected control groups. Finally, our results have been confirmed and cited by a more recent MA focusing on a different subject, but coming to identical conclusions in a supplementary analysis parallel to its main object of interest (5HTTLPR and stressful events in ED(46). The main limitations of the present study were: (1) the lack of studies controlling for comorbid anxiety, mood disorders, and suicidal behaviour, all possibly associated with 5HTTLPR polymorphism(47, 48); (2) few studies included Asian patients, precluding a better understanding of the relationship between 5HTTLPR polymorphisms and ED in that ethnic group; (3) there were frequent definitions of control groups as not being affected by ED, without clear exclusion of other organic or psychiatric diseases; (4) there was a lack of studies to date reporting data using the new diagnostic criteria using DSM-5(49), and (5) only two studies compared ED with obesity, which renders these results preliminary. In conclusion, the present meta-analysis did not confirm results from previous single studies hypothesising an additive major role of 5HTTLPR polymorphism for the risk of developing an ED. However, data provided by the present meta-analysis cannot rule out a possible small additive effect of 5-HTTLPR (that could be demonstrated only in very large samples), or an interactive effect. Thus, future studies should be designed to overcome the limitations of available studies, including a better definition of healthy control groups, the use of triallelic models, assessment of the effects of psychiatric comorbidity and ethnic differences, as well as providing data about possible environmental risk factors – such as stressful and traumatic events – that might interact with 5-HTTLPR polymorphism in increasing the risk for developing an ED.

Table 1. Characteristics of included studies.

Study/country	AN/BN/control group diagnostic criteria	Control group (Y/N/HC/other disease)	N AN/ BN	N Control group	Age AN/BN	Age control	BMI AN/BN (kg/m^2)	BMI control (kg/m^2)	Onset age (years)	Measurement method/	Comorbidity	Medications	Personality or symptom scale or other clinical data	Genotype in Hardy–Weinberg equilibrium
With control group														
Hinney et al. (1997) Germany	CIDI, DSM IV	Underweight students; obese cohorts combined	AN 96	Obese = 385 Underweight = 112	F: 16.6 ± 3.4; M:15.3 ± 0.9	Under-weight: F:24.7 ± 3.9; M:26.1 ± 4.1 Obese F 29.57 ± 6.63; M 29.73 ± 5.3	BMI F:14.5 ± 1.5; M:13.9 ± 2.0	Underweight:- F:17.6 ± 0.8; M:19.0 ± 1.0 OBESE F 30.5 ± 5.3; M 33.6 ± 4.27	–	PCR, blood, biallelic	–	–	Three factor eating questionaire	Yes
Di Bella et al. (2000) Italy	DSM IV	HC	AN 56 (19 R e 37 BP) BN 50	120	–	–	–	–	–	Blood, biallelic	–	–	–	Yes, except BN with lower I/I genotype.
Fumeron et al. (2001) France, Caucasians	DSM IV	HC overweight subjects	65F 2M; AN	HC: 84F, 64M Overweight: 215F, 143M	24.3 ± 6.5	HC: 41.2 ± 4.1 Over-weight: 41.5 ± 10.3	13.6 ± 1.9	HC: 22.6 ± 2.1 Overweight: 32.5 ± 4.6		PCR, blood, biallelic	–	–	–	Yes
Sundaramurthy et al. (2000) UK, Caucasian	DSM IV	HC	AN 138	90	18.1	30.28	13.73 (minimum average BMI)	22.02	–	PCR, blood, biallelic	–	–	–	Yes
Lauzurica et al. (2003) Spain	DSM-IV	HC	BN 102 (36 previous AN)	107	BN 21.4 ± 6, prev AN 20.5 ± 6	31.1 ± 10.34	BN 22.5 ± 5, prev AN 21.09 ± 3	23 ± 3.1	–	Blood, PCR, biallelic	No major psychiatric comorbidity	–	SCID-I, BITE, others	Yes
Matsushita et al. (2004) Japan	DSM IV	HC with EAT 26 score<20, or minimum BMI<17.5	F. 77 AN; 118 BN; 14 EDNOS	290F	25.8 ± 6.6	25.3 ± 8.0		BMI>17.5	–	Blood, WBkit PCR, biallelic	–	–	–	Yes
Urwin et al. (2005) Australia	DSM-IV	Mothers and fathers	AN 114	106 trios, 8 duos	16.7 ± 3.46	–	Min 14.6 ± 1.7	–	1.99 ± 1.97 before hospitalization	PCR, biallelic	–	–	–	Yes
Monteleone et al. (2006a) Italy	DSM IV	HC	F 77 BED	61 F Caucasian	–	–	–	–	–	Blood PCR biallelic	–	–	SCID I e II	Yes
Monteleone et al. (2006b) Italy	DSM IV	HC	125 F BN P	94 HC F	18–44 range	18–41 range	–	–	–	Blood, PCR, biallelic	–	–	SCID I, II, TCI-R	Yes
Rybakowski et al. (2006) Poland	DSM IV	HC	132 AN F	93 HC F	17.6 ± 2.9	20.9 ± 1.6				Blood PCR, biallelic	–	–	TCI	Yes
Martásková et al. (2009) Czech Republic	DSM-IV	HC	AN 72	65	25.39 ± 6.18	25.76 ± 5.12	14.65 ± 1.38	20.69 ± 1.85	–	Blood, PCR, biallelic				Yes
Steiger et al. (2009) Canada, Caucasian	DSM-IV	Normal-eaters	AN-R 9, AN-BP 8, BN-NP 9, BN-BP 99, BN EDNOS 47, AN EDNOS 13.	93	25.92 ± 7.05	24.43 ± 6.24	21.39 ± 3.89	22.00 ± 2.55	–	Blood, PCR, biallelic, triallelic	8 control Axis I diagnosis.	77 ED, 1 control	EDE, DAPP, BIS, CES-D, SCID-I, DIS4	Yes
Ehrlich et al. (2010) Germany	DSM-IV, ICD-10	HC	acAN 58, recAN 36	58	acAN 17.4 ± 2.7, recAN 19.5 ± 3.3	18.4 ± 2.9	acAN 15.2 ± 1.4, recAN 20.7 ± 2.2	21.6 ± 2	–	Blood, PCR, biallelic.	No major psychiatric comorbidity, apart from depressive or anxiety disorders.	No psychotropic medications	SCL-90R, SIAB-EX, EDI-2	Yes

(continued)

Table 1. Continued

Study/country	AN/BN/control group diagnostic criteria	Control group (Y/N/HC/other disease)	N AN/ BN	N Control group	Age AN/BN	Age control	BMI AN/BN (kg/m^2)	BMI control (kg/m^2)	Onset age (years)	Measurement method/	Comorbidity	Medications	Personality or symptom scale or other clinical data	Genotype in Hardy–Weinberg equilibrium
Karwautz et al. (2011) Austria, UK, Spain, Caucaisians	DSM-IV	Healthy sisters	AN-R 58, AN-BP 70	128	25.35 ± 8.1	25.9 ± 8.7	18.4 ± 2.3	22.6 ± 7.9	16.5 ± 4.2	Blood, PCR, biallelic	–	–	Environmental domanis (parenting style, disruptive events, interpesonal problems, dieting environment)	Yes
Castellini et al. (2012) Italy, Caucasian	DSM-IV	HC	AN-BP 65, AN-R 48, BN-BP 88	150	26.54 ± 7.55 AN, 28.37 ± 7.59 BN	26.07 ± 20.74	16.56 ± 2.6 AN, 23.6 ± 5.63 BN	–	–	Blood, PCR, biallelic	AN 54.9%, BN 36.7%	Antidepressants AN 37.2%, BN 29.5%, Anxiolytics AN 22.1%, BN 18.2%.	EDE-Q, BDI, STAI	Yes
BIO.VE.D.A. group Italy, Caucasian	DSM-IV	HC	ED 735 (AN lifetime 526, BN lifetime 341)	241	ED 25.56 (8.97); AN lifetime 25.01 (8.94); BN lifetime 26.45 (8.36)	25.84 (5.71)	ED 19.02 (4.27) AN lifetime 17.47 (3.07); BN lifetime 21.64 (4.21)	21.59 (2.93)	–	Blood and saliva, PCR biallelic and triallelic	No comorbidity	–	–	Yes
Sixteen studies	*DSM-IV 16/16*	*12 HC, 2 obese, 1 normal-eaters, 1 healthy sisters, 1 EAT-26 < 20 + min BMI > 17.5, 1 parents*	*AN 1679, BN 979; total 2749 with EDNOS and BED*	*1964HC, 743 obese; total 2707*	*23.83 ± 8*	*29.74 ± 11.12 yo*	*18.79 ± 4.55*	*26.29 ± 6.79*	*Reported in 2/16 studies*	*14/16 bialleic only, 2/16 Bi + triallelic*	*Reported in 5/16 studies*	*Reported in 3/16 studies*	*Reported in 9/16 studies*	*15/16*
No control group														
Frieling et al. (2006) Germany	DSMIV	N	ANR 7, ANP 14, BNP 21, BNNP 3	–	AN 26.83 ± 10.43; BN 25.84 ± 7.96	–	AN 15.7 ± 1.86, BN 22.52 ± 2.56	–	–	Blood, PCR, biallelic			SCID I, II, EDI2	Yes
Ribasés et al. (2006) Spain, Caucasian	DSM IV	N	AN 46 F; BN 36 F	–	AN 24.6 ± 4.4; BN 25.1 ± 6.5	–	–	–	AN: 17.1 ± 3.3; BN: 18.6 ± 4.6	PCR biallelic	–	–	SCID I e SCL 90-R	Yes
Steiger et al. (2008a) Richardson et al. (2008) Canada	DSM IV	N	69 BN P F; 4 BN NP F; 17 BN NAS F	–	25.29 ± 6.4	–	22.39 ± 2.67	–	–	Blood, PCR, biallelic and triallelic	Depression, anxiety, alcohol or drug abuse.	41 on medications psico	EDE, EAT-26, SCID-I, CAPS, DAPBQ, CTI	Yes

(continued)

Table 1. Continued

Study/country	AN/BN/control group diagnostic criteria	Control group (Y/N/HC/other disease)	N AN/ BN	N Control group	Age AN/BN	Age control	BMI AN/BN (kg/m^2)	BMI control (kg/m^2)	Onset age (years)	Measurement method/	Comorbidity	Medications	Personality or symptom scale or other clinical data	Genotype in Hardy–Weinberg equilibrium
Steiger et al. (2008b) Canada, 95 white European; 2 Latin American; 1 mix asian	DSM IV	N	72 BN P, 3 BN NP, 23 BN NOS	–	26.81 ± 7.15	–	22.38 ± 3.90	–	–	Blood, PCR, biallelic and triallelic			CES- D, BASIS-32, BIS-11	Yes
Steiger et al. (2011) Canada, Germany, Caucasian	DSM-IV	N	AN-R 63, AN-BP 59, BN 221, EDNOS 56	–	25.04 ± 5.93 (<50)	–	19.88 ± 4.32 (<35)	–	–	Blood, PCR, triallelic	–	–	EDE, SIAB-EX, SCID-II Self-harm	Yes
Thaler et al. (2013) Canada, 95.3% Caucasian	DSM-IV	N	177 BN purge, 14 BN non purge, 82 EDNOS	–	25.91 ± 6.62	–	22.62 ± 3.84	–	–	Blood, PCR, triallelic	–	127 on medications	EAT-26, DAPP, BIS, SCID-II (hierarchical linear regression)	Yes
Six studies	*DSM-IV 6/6*	*N*	*AN 189 BN 660, EDNOS 138; total 987*	*N*	*25.51 ± 11.03 yo*	*N*	*21.20 ± 4.20*	*N*	*Reported in 2/6 studies*	*2/6 biallelic, 4/6 biallelic and triallelic*	*Reported in 1/6*	*Reported in 2/6*	*Reported in 6/6*	*6/6.*
OVERALL Total 22 studies	All DSM-IV	*12 HC, 2 obese, 1 normal-eaters, 1 healthy sisters, 1 parents, 1 EAT-26 $\leq$ 20 ± min BMI $\geq$ 17.5.6 no controls*	AN 1868; BN 1639; Total ED 3736	*1964HC, 743 obese; total 2707*	24.27±-8.9 yo	*29.74 ± 11.12 yo*	19.43 ± 4.58	*26.29 ± 6.79*	Reported in 4/22 studies	16/22 biallelic, 6/22 triallelic	6/22	5/22	15/22	21/22

AN, anorexia nervosa; AN-BO, AN bulimic purgative; ANR, AN restricter; BASIS-32, Behaviour and Symptom Identification Scale; BIS, Barratt Impulsiveness Scale; BN, bulimia nervosa; CAPS, Clinical-Administered Post-Traumatic Stress Disorder Scale; CES-D, Centre for Epidemiological Studies Depression; CTI, childhood trauma interview; DAPP, dimensional assessment for personality pathology; DIS4, Diagnostic Interview Schedule, Version IV; EAT-26, Eating Attitude Test; EDE, eating disorder examination; EDNOS, eating disorder not otherwise specified; HC, healthy control; PCR, polymerase chain reaction; SCID-II, Structured Clinical Interview for DSM-IV Axis II diagnoses; SCL-90, Symptom Checklist 90 – Revised; SIAB-EX, Structured Interview for Anorexic and Bulimic Syndromes; TCI-R Temperament and Character Inventory Revised; yo, years old.

Table 2. High and low-functioning genotype and allele frequencies within patients and controls.

Comparison	Number of studies	Studies	Participants or alleles	Statistical method	Effect estimate [95% CI]*
	Within patients and within controls				
Biallelic genotype low vs. high ED	21	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Ehrlich et al. 2010; Frieling et al. 2006; Fumeron et al. 2001; Hinney et al. 1997; Karwautz et al. 2011; Lauzurica et al. 2003; Martaskova et al. 2009; Matsushita et al. 2004; Monteleone et al. 2006a; Monteleone et al. 2006b; Ribases et al. 2008; Richardson et al. 2008; Steiger et al. 2008b; Rybakowski et al. 2006; Steiger et al. 2008a; Steiger et al. 2009; Sundaramurthy et al. 2000; Thaler et al. 2013; Urwin et al. 2005)	3024	Odds ratio (M-H, random. 95% CI)	5.79[4.10,8.17] $P<0.00001$
Triallelic genotype low vs. high ED	6	(BIO.VE.D.A. group; Rybakowski et al. 2006; Steiger et al. 2008a; Steiger et al. 2009; Steiger et al. 2011; Thaler et al. 2013)	1771	Odds ratio (M-H, random. 95% CI)	7.90 [6.50. 9.58]; $P<0.00001$
Biallelic vs. triallelic genotype ED	*5*	*(BIO.VE.D.A. group; Richardson et al. 2008; Steiger et al. 2008b; Steiger et al. 2008a; Steiger et al. 2009; Thaler et al. 2013)*	*1381*	*Biallelic: 924 low 457 high. Triallelic: 1012 low 369 high*	
Biallelic genotype low vs. high controls	16	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Ehrlich et al. 2010; Fumeron et al. 2001; Hinney et al. 1997; Karwautz et al. 2011; Lauzurica et al. 2003; Martásková et al. 2009; Matsushita et al. 2004; Monteleone et al. 2006a; Monteleone et al. 2006b; Rybakowski et al. 2006; Steiger et al. 2009; Sundaramurthy et al. 2000; Urwin et al. 2005)	2146	Odds ratio (M-H, random, 95% CI)	4.95 [3.09, 7.92]$P<0.00001$
Triallelic genotype low vs. high controls	2	(BIO.VE.D.A. group; Steiger et al. 2009)	334	Odds ratio (M-H, random, 95% CI)	18.80 [3.81, 92.71]; $P=0.0003$
Biallelic vs. triallelic genotype controls	*2*	*(BIO.VE.D.A. group; Steiger et al. 2009)*	*334*	*Biallelic: 233 low 101 high. Triallelic: 260 low 73 high*	
Biallelic alleles low vs. high ED	14	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Frieling et al. 2006; Fumeron et al. 2001; Hinney et al. 1997; Martásková et al. 2009; Matsushita et al. 2004; Monteleone et al. 2006a; Monteleone et al. 2006b; Rybakowski et al. 2006; Steiger et al. 2009; Sundaramurthy et al. 2000; Urwin et al. 2005)	4566	Odds Ratio (M-H, random, 95% CI)	0.88 [0.55, 1.42]; $P=0.60$; without Matsushita 0.69 [0.56, 0.85]; $P=0.0007$
Triallelic alleles low vs. high ED	3	(BIO.VE.D.A. group; Steiger et al. 2009, Steiger et al. 2011)	2620	Odds ratio (M-H, random, 95% CI)	1.03 [0.79, 1.34]; $P=0.99$
Biallelic vs. triallelic alleles ED	*2*	*(BIO.VE.D.A. group; Steiger et al. 2009)*	*1840*	*Biallelic: 785 low 1055 high. Triallelic: 890 low 940 high*	
Biallelic alleles low vs. high controls	13	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Fumeron et al. 2001; Hinney et al. 1997; Martásková et al. 2009; Matsushita et al. 2004; Monteleone et al. 2006; Monteleone et al. 2006b; Rybakowski et al. 2006; Steiger et al. 2009; Sundaramurthy et al. 2000; Urwin et al. 2005)	3326	Odds ratio (M-H, random, 95% CI)	0.77 [0.42, 1.4]; $P=0.43$; without Matsushita 0.62 [0.46, 0.85]; $P=0.003$
Triallelic alleles low vs. high controls	2	(BIO.VE.D.A. group; Steiger et al. 2009)	666	Odds ratio (M-H, random, 95% CI)	1.28 [0.59, 2.77]; $P=0.54$
Biallelic vs. triallelic allele controls	*2*	*(BIO.VE.D.A. group; Steiger et al. 2009)*	*666*	*Biallelic: 296 low 372 high. Triallelic: 340 low 326 high*	

*Effect estimate >1 indicates higher frequency of low-functioning genotype or alleles.
AN, anorexia nervosa; BN, bulimia nervosa; ED, eating disorder.

Table 3. Low-functioning genotype and allele frequencies in patients vs. controls.

Comparison	Number of studies	Studies	Participants (patient vs. HC) or alleles	Statistical method	Effect estimate [95% CI]*
ED. AN. BN vs. controls					
Biallelic					
Low-functioning genotype SS or SL (recessive model)					
ED vs. controls	16	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Ehrlich et al. 2010; Fumeron et al. 2001; Hinney et al. 1997; Karwautz et al. 2011; Lauzurica et al. 2003; Martaskova et al. 2009; Matsushita et al. 2004; Monteleone et al. 2006a; Monteleone et al. 2006b; Rybakowski et al. 2006; Steiger et al. 2009; Sundaramurthy et al. 2000; Urwin et al. 2005)	2555 vs. 1936	Odds ratio (M-H, random, 95% CI)	1.07 [0.87, 1.33]; P=0.51
AN vs. controls (Figure 2).	13	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Ehrlich et al. 2010; Fumeron et al. 2001; Hinney et al. 1997; Karwautz et al. 2011; Lauzurica et al. 2003; Martaskova et al. 2009; Matsushita et al. 2004; Rybakowski et al. 2006; Sundaramurthy et al. 2000; Urwin et al. 2005)	1637 vs. 1688	Odds ratio (M-H, random, 95% CI)	1.13 [0.96, 1.32]; P=0.15
BN vs. controls	6	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Lauzurica et al. 2003; Matsushita et al. 2004; Monteleone et al. 2006b)	824 vs. 1002	Odds ratio (M-H, random, 95% CI)	1.29 [0.78, 2.14]; P=0.32
Low-functioning genotype SS (dominant model)					
ED vs. controls	16	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Ehrlich et al. 2010; Fumeron et al. 2001; Hinney et al. 1997; Karwautz et al. 2011; Lauzurica et al. 2003; Martaskova et al. 2009; Matsushita et al. 2004; Monteleone et al. 2006; Monteleone et al. 2006b; Rybakowski et al. 2006; Steiger et al. 2009; Sundaramurthy et al. 2000; Urwin et al. 2005)	2555 vs. 1936	Odds ratio (M-H, random, 95% CI)	1.10 [0.89, 1.37]; P=0.37
AN vs. controls	13	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Ehrlich et al. 2010; Fumeron et al. 2001; Hinney et al. 1997; Karwautz et al. 2011; Lauzurica et al. 2003; Martaskova et al. 2009; Matsushita et al. 2004; Rybakowski et al. 2006; Sundaramurthy et al. 2000; Urwin et al. 2005)	1637 vs. 1688	Odds ratio (M-H, random, 95% CI)	1.27 [1.01, 1.60]; P=0.04; **without Matsushita 1.19 [0.95, 1.50]; P=0.13**
BN vs. controls	6	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Lauzurica et al. 2003; Matsushita et al. 2004; Monteleone et al. 2006b)	824 vs. 1002	Odds ratio (M-H, random, 95% CI)	1.03 [0.73, 1.46]; P=0.85
Low-functioning alleles					
ED vs. controls	13	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Fumeron et al. 2001; Hinney et al. 1997; Martaskova et al. 2009; Matsushita et al. 2004; Monteleone et al. 2006a; Monteleone et al. 2006b; Rybakowski et al. 2006; Steiger et al. 2009; Sundaramurthy et al. 2000; Urwin et al. 2005)	4487 vs. 3326	Odds ratio (M-H, random, 95% CI)	1.05 [0.88, 1.26]; P=0.56
AN vs. controls (Figure 3).	10	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Fumeron et al. 2001; Hinney et al. 1997; Martaskova et al. 2009; Matsushita et al. 2004; Rybakowski et al. 2006; Sundaramurthy et al. 2000; Urwin et al. 2005)	2783 vs. 2830	Odds Ratio (M-H, Random, 95% CI)	**1.19 [1.01, 1.39]; p = 0.03; Without Matsushita 1.15 [0.98, 1.3]; p = 0.09**
BN vs. controls	5	(BIO.VE.D.A. group; Castellini et al. 2012; Di Bella et al. 2000; Matsushita et al. 2004; Monteleone et al. 2006b)	1444 vs. 1790	Odds ratio (M-H, random, 95% CI)	1.10 [0.76, 1.59]; P=0.62
Triallelic					
Low-functioning genotype					
ED vs. controls	2	(BIO.VE.D.A. group; Steiger et al. 2009)	920 vs. 333	Odds ratio (M-H, random, 95% CI)	0.20 [0.01, 3.62]; P=0.27
Low-functioning alleles					
ED vs. controls	2	(BIO.VE.D.A. group; Steiger et al. 2009)	1840 vs. 666	Odds ratio (M-H, random, 95% CI)	0.92 [0.77, 1.10]; P=0.34

AN: anorexia nervosa; BN: bulimia nervosa; ED: eating disorder.

Table 4. BIO.VE.D.A. Group 5HTTLPR frequencies in subjects with eating disorders.

Diagnosis	Genotype							Allele Frequency				Age (SD)	M, F
	La/La	La/Lg	Lg/Lg	S/La	S/Lg	S/S	Total[a]	Lg	La	S	Total[a]		
AN lifetime	140 (26.6%)	29 (5.6%)	3 (0.5%)	235 (44.8%)	23 (4.3%)	96 (18.2%)	526	58 (5.6%)	544 (51.7%)	450 (42.7%)	1052	25,01 (8.94)	10, 516
BN lifetime	105 (30.8%)	12 (3.5%)	1 (0.3%)	140 (41.1%)	22 (6.4%)	61 (17.9%)	341	36 (5.3%)	362 (53%)	284 (41.7%)	682	26,45 (8.36)	16, 325
Healthy controls	61 (25.3%)	16 (6.6%)	2 (0.8%)	111 (46.1%)	5 (2.1%)	46 (19.1%)	241	25 (5.2%)	249 (51.6%)	208 (43.2%)	482	25,84 (5.71)	9, 232
Eating disorders	210 (28.6%)	34 (4.7%)	3 (0.4%)	322 (43.8%)	37 (5%)	129 (17.5%)	735	77 (5.2%)	776 (52.9%)	617 (41.9%)	1470	25,56 (8.97)	22, 713

AN, anorexia nervosa; BN, bulimia nervosa; ED, eating disorder.

[a]The total number of subjects with eating disorders is smaller than ther sum of subjects with AN and BN, as life-time AN and life-time BN was the defining criterion for subgroup membership, resulting in a subgroup of patients with both life-time AN and life-time BN.

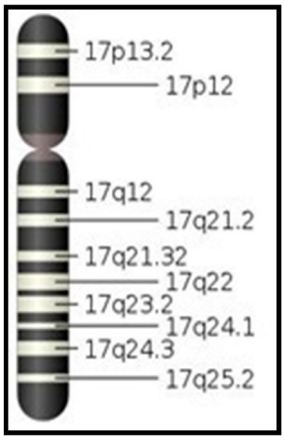

Figure 1. Chromosome 17.

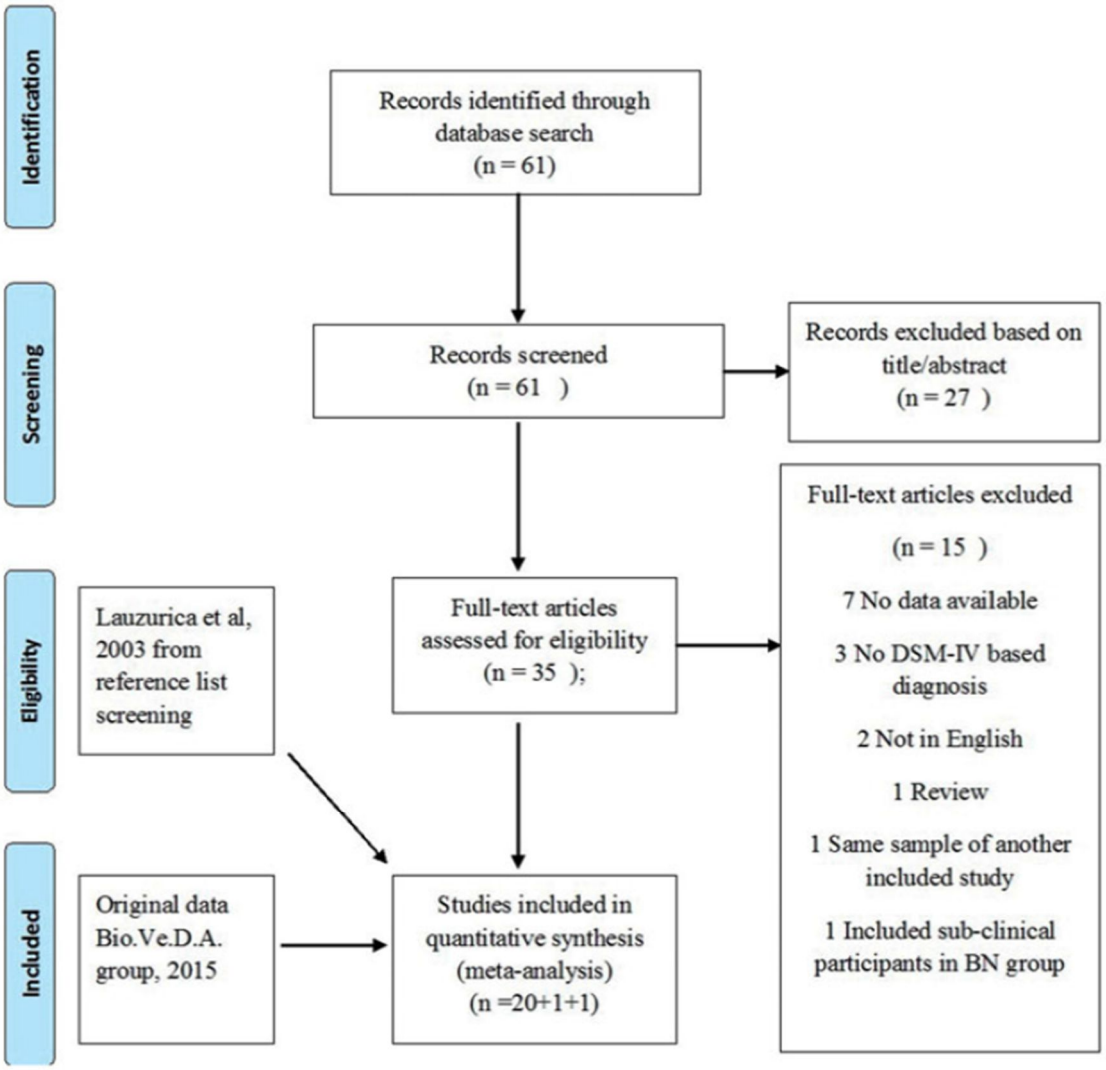

Fig 2. PRISMA flow diagram of study selection process in 5HTTLPR and Eating Disorders meta-analysis.

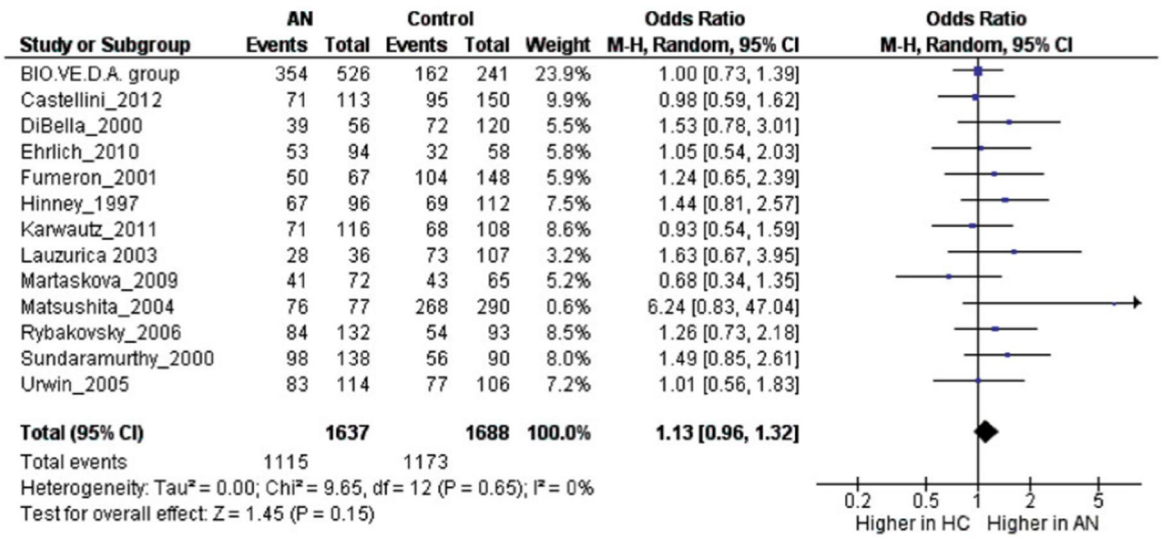

Fig 3. Bi-allelic low functioning genotype frequency in anorexia nervosa vs healthy controls.

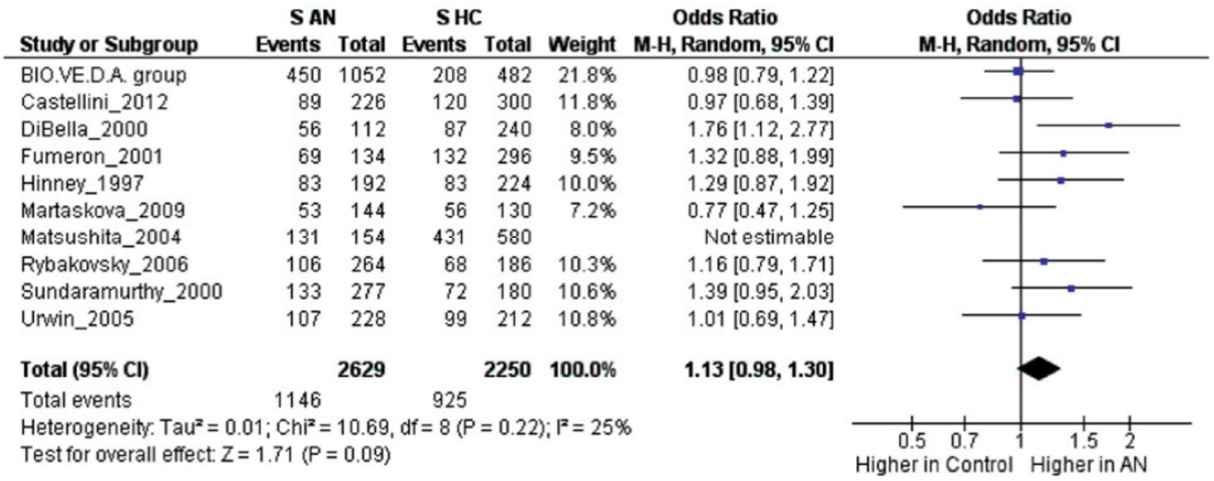

Fig 4. Bi-allelic low functioning allele frequency in anorexia nervosa vs healthy controls.

3. STRESSFUL EVENTS AND 5HTTLPR IN EATING DISORDERS

3.1 INTRODUCTION

Beyond genetics, environmental factors have a role in all medical conditions, and in particular in psychiatry where reality perception, interpersonal attitude, impulsivity, avoidance, perseverance, cognitive performance, behavior, resilience and thought processes can all interact with environment in a reciprocal way, and determine one individual's subjective perception of traumatic or non-traumatic events. Stressful events (SE) have been extensively studied in their role of potentially determining several psychiatric disorder, and have been associated with major depressive disorder (MDD)(50, 51), and bipolar disorder (BD) (51) among others. Several hypotheses have been formulated about the putative mechanism underlying the link between SE and psychiatric disorders. Among other psychological factors, attachment style has been proposed as a mediator between traumatic events and development of depression, and has also been proposed as a potential therapeutic target in the pathogenetic pathway to MDD (52). From a biological perspective, early traumatic events may influence the onset of both MDD(53) and BD(54) through an epigenetic mechanism, which in turn may reciprocally interact with the hypothalamic-pituitary-adrenal axis dysregulation which is known in MDD and BD.

Regardless SE and traumatic events, depression has also been associated with 5HTTLPR low functioning polymorphism (9). Such association has then been tested and confirmed beyond environmental factors or genetics per se, providing evidence of a gene by environment mechanism involving 5HTTLPR polymorphism and SE as causal factor for affective disorders(55, 56). As regards Eating Disorders (ED), several pieces of evidence suggest a role of SE in the pathogenesis of these severe conditions(57, 58). Conversely, the low functioning 5HTTLPR S allele does not seem to be associated with ED, according to the meta-analysis we published in World Journal of Biological Psychiatry(28), recently confirmed by a systematic review. This latter recent systematic review by Rozenblat et al (46) additionally focused on ED risk and 5HTTLPR by SE interaction. It showed that two studies found a 5HTTLPR by traumatic life events association with disordered eating, but only one found an association with Anorexia Nervosa (AN) diagnosis (40, 59, 60). Moreover in this latter study (40) the 5HTTLPR by SE association only included risky parenting style as SE, excluding milder trauma definitions, which could not interact with 5HTTLPR polymorphism instead. Furtherly complicating the big picture, considering only sexual abuse as SE, one study found ED symptoms, as reated by specific scales, depending on a 5HTTLPR by SE interaction, while other authors did not replicate the same finding (15). In line with the aforementioned systematic review (46) we aimed to

investigate the association of 5HTTLPR and SE with ED beyond the two risk factors separately (see above), testing a gene by environment model on ED similarly to what has been tested in depression.

3.2 METHODS

Sample and Questionnaires. We included 141 controls and 686 patients with life-time diagnosis of ED. Patients were divided in 181 AN Restricters, 38 AN Purging type, 31 Bulimia Nervosa (BN) Non Purge, 159 BN Purging type, 277 Eating Disorder Non Otherwise Specified (EDNOS). Patients have been recruited in 5 Eating Disorders specialized centers in Veneto region in the context of Bio.Ve.D.A. (BIobanca VEneta per i Disturbi Alimentari) (see above). Inclusion criteria were a life-time diagnosis of AN, BN, EDNOS according to DSM-IV(32), age older than 14 years old, patients' and parental (if less than 18 years old) informed consent. Exclusion criteria were comorbidity with organic or other psychiatric disorders. The presence of stressful events has been investigated in all participants through a list including partner separation or divorce, study or work related failures, city moving, environment, social or familiar conflictuality, death of significant others, abortion, violence or sexual abuse, severe medical sickness. Their date of occurrence and subjective severity scoring from 1(mild) to 5 (severe) were collected. The DNA sampling has been performed as described above (Chapter 2).

Statistical analysis. We investigated the association between S-allele and ED or AN or BN using chi-square test ($\chi 2$), in order to understand if 5-HTTLPR polymorphism had a pathogenetic role by his own (see above). Gene by environment hypothesis has been preliminarily studied dividing the sample in patients with and without S allele, through $\chi 2$ and Mantel-Haenszel; independent variables were stress before age 12, and 5HTTLPR low functioning allele, while dependent variable was the presence of an ED. The same analysis has been performed considering stress before 18 years instead of 12 years age. In addition, we tried to assess if mild, moderate, and severe SE differently contributed to ED risk, in both groups with and without S allele, via a logistic regression to test the 5HTTLPR by SE interaction according to the multiplicative model. We tested if S-allele increased perception of severity of stressful events. Finally we tested a 5HTTLPR by SE additive interaction model using the Relative Excessive Risk due to Interaction (RERI) (61).

3.3 RESULTS.

Participants. Mean BMI of the 117 HC was 21.76 (3.12) Kg/m^2, mean age 25.42(5.75) years old; mean BMI of 686 patients with ED was 19.14 (3.85) Kg/m^2, mean age was 24.89 (8.84) years old. In patients with AN minimum BMI was 15.11 (1.96) Kg/m^2, while in BN minimum BMI was 20.12 (3.1) Kg/m^2.

Stressful events and 5HTTLPR polymorphism. No significant association was found.

Childhood stressful events, and S allele. Our results show an increased risk of both AN (OR=1.84, 95%CI 1.17-2.91, p=0.009) and BN (OR=2.08, 95%CI 1.30-3.35, p=0.002) in subjects experiencing severe stressful events in childhood, regardless the 5HTTLR genotype. Dividing the sample according to S allele, when the same analyses were run in patients without S allele, no such association was found, which in turn was confirmed in patients with S allele (tab 5).

*Life-time stressful events according to severity, and S allele***.** Our results show an increased association of ED, and of AN and BN separately, with lifetime stressful events. The relationship was significant regardless the 5HTTLPR genotype, and also considering patients with or without S allele separately. More severe stressful events conferred higher risk of ED.

Correlations. Regardless 5HTTLPR polymorphism, minimum life-time Body Mass Index (BMI), age of onset, EDI drive for thinness, body shape concerns, and ineffectiveness, self-esteem, alongside with obsessive-compulsive, depression and anxiety comorbidity correlated with the number of SE experience. Age of onset, ineffectiveness, self-esteem, alongside with obsessive-compulsive, depression and anxiety comorbidity correlated with the number of childhood SE. When considering patients without S allele, only age of onset, body shape concerns, and ineffectiveness remained significantly correlated with life-time or childhood SE, while considering those with S allele minimum life-time BMI, age of onset, EDI drive for thinness, body shape concerns, and ineffectiveness, self-esteem, alongside with obsessive-compulsive, depression and anxiety comorbidity remained significant.

5HTTLPR by SE and risk of ED. According to logistic regression the risk of ED according to severity of stress was less increased in patients with no S allele (β=4.81, 95%CI 1.68-13.74, p=0.003), compared to those with S allele (β=9.17, 95% CI 4.02-20.89, p<0.001), and the same direction and magnitude of results were replicated when analyzing separately patients with AN and BN. However we did not find any 5HTTLPR by SE multpicative or addidtive interaction.

3.4 DISCUSSION

Our results add to the available evidence about 5HTTLPR by interaction SE model in ED, recently reported in an important recent systematic review and meta-analysis (46). We found a significant correlation of specific ED psychopathologic symptoms, such as drive for thinness, body shape concerns, perceived ineffectiveness, and of obsessive and depressive comorbidity with S allele, in presence of SEs. Also, wWhile 5HTTLPR and milder or single life events are not individually

associated with ED, suffering from childhood SE and more severe SEs increases the ED risk. Moreover, while having a low functioning allele seems to expose patients who suffered from severe SE to an increased risk of ED, such an interactioin has not been confirmed according to either the gene by environment interaction multiplicative or additive model in ED. Such a different results from the above emntined recent systematic review and MA, could be due to the fact that we included full diagnoses ED only according to DSM-IV(32), and not only to possibly subclinical subjects. Thus, while ED symptoms could be linked to a 5HTTLPR by SE multiplicative or additive interaction, possibly in comorbidity with other psychiatric disorders, in particular depressive disorder, ED *tout court* do not seem to have such an interaction underpinning its pathogenesis. We nonetheless confirm that 5HTTLPR does not interact with SE according to any additive model, as previously suggested from Rozenblat(46). This moderates any conclusion about a real gene by environment interaction involved in the pathogenetic pathway of ED, since the additive interaction model measures, RERI, is supposed to better explain and to be more sensitive to identifying gene by environment interactions in biology (additive model) compared with other models.

Beyond any causal inference, these data can be relevant at a patient level. For example, if clinicians know that a patient is an S-allele carrier, depressive and obsessive comorbid conditions should be suspected, and patients should be monitored for eventual emerging of such disorders beyond the acute phase of any ED. Moreover, clinicians should account for those psychopathologic dimensions (drive for thinness, body shape concerns, ineffectiveness) that could persist even if weight and behavioral pathological phenomena required for an ED diagnosis are no more formally satisfied. We suggest that with such an approach, ED relapse, which is a common event in AN and BN, could eventually be prevented or at least moderated. Also, since comorbid conditions obstacle and make ED more resistant to classic therapeutic tools, a proper detection of eventual depressive and obsessive symptomatology should be warranted. Finally, in the context of cognitive-behavioral psychotherapy or family therapy, if thanks to the 5HTTLPR genotyping a therapist since the very beginning of the treatment suspects dominant specific psychopathologic domain, we suggest better or faster results could eventually be achieved. However such an effect of the use of 5HTTLPR gemotyping in clinical activity should be investigated with ad hoc designed studies.

Our study has several strength points. First of all the sample size is large and provides reliable and solid results. Secondly, we assessed SE with a structured interview, minimizing the subjective component in reporting ESs. Third, we also excluded the possibility to have a biased recall of SE in low or high functioning 5HTTLPR genotypes, since we did not find any association between S allele frequency and number of SEs. Fourth, we included both full criteria standardized diagnosed ED, and

ED psychopathology measures, such as EDI as well. Finally, our results are at essentially in line with the more recent and large MA investigating the 5HTTLPR by SE interaction model in ED, at least as regards the absence of an additive interaction.

Several limitations should be mentioned as well. We only included female subjects; however this is due to gender asymmetric distribution of ED. Secondly, we did not distinguish between restrictive and purging AN, or between purging or non-purging BN. Also, we did not consider Binge Eating Disorder at all, since we mainly focused on AN and BN. Finally, our SE related information were collected with a retrospective design. Even if we tried to minimize it, the retrospective design could have introduced some recall-biased information.

In conclusion our results suggests that 5HTTLPR polymorphism may moderate the effect of SEs in increasing the risk of ED onset. Moreover the absence of S allele seemed to moderate the influence of SEs on ED severity, anxious, depressive and obsessive symptoms. However when we tested a multiplicative interaction, and an additive model, which is considered to be more representative of a real-world gene by environment interaction, the 5HTTLPR by SE interaction was not confirmed.

		Any Stressful Event	OR	IC 95%
Controls (141)	S Allele =0 (42)	21,4% (9)		
	S Allele =1 (99)	20,2% (20)		
Eating Disorders (679)	S Allele =0 (198)	34,8% (69)	1,96 (p=0,096)	0,89 - 4,33
	S Allele =1 (481)	**34,3% (165)**	**2,06 (p=0,007)**	**1,22 - 3,49**
AN (422)	S Allele =0 (112)	36,6% (41)	2,12 (p=0,077)	0,92 - 4,86
	S Allele =1 (310)	**31,0% (96)**	**1,77 (p=0,040)**	**1,03 - 3,06**
BN (288)	S Allele =0 (87)	31,0% (27)	1,65 (p=0,257)	0,69 - 3,92
	S Allele =1 (201)	**36,3% (73)**	**2,25 (p=0,005)**	**1,28 - 3,99**

Tab 5. Stressful events and risk of Eating Disorders, according to 5HTTLPR polymorphism.

4. NEUROPSYCHOLOGICAL PERFORMANCE AND 5HTTLPR IN EATING DISORDERS

4.1 INTRODUCTION

Several psychiatric disorders, such as major depressive disorder (MDD), bipolar disorder (BD), and schizophrenia are associated with deficitary cognitive functions. More in particular MDD with psychotic symptoms has shown a deficit in verbal and visual learning, and executive functions(62), as depression with melancholic features did(63). Moreover both BD and schizophrenia are associated with poor cognitive performance, more severly in schizophrenia, and in patients with early onset of disease, in several domains(64). Patients with Anorexia Nervosa (AN) have shown a lower performance in several neuropsychological functions compared to healthy controls as well. A wide range of cognitive functions performance have been questioned in eating disorders (ED); cognitive flexibility, set-shifting, central coherence and even theory of mind seems to be impaired specifically in AN(65). In this context we recently showed that patients affected by ED have a lower performance at Cognitive Bias Task (66) independently from general psychopathology, education, handedness and ED diagnostic subtypes, compared to age and gender matched subjects without psychiatric condition. Also, our research group showed that patients with AN had poorer central coherence (defined as the ability to prioritize the "big picture" instead of small details), and poorer set-shifting (defined as the ability to update decisions according to environmental updated rules) compared to healthy controls (HC), and that this defect also involved patients' unaffected siblings (67), possibly indicating a cognitive endophenotype of AN. Also patients with bulimia nervosa (BN) appear to have a lower decision making performance as measured by Iowa Gambling Task, but the differences with HCs were more pronounced in BN group with previous AN (68). Similarly, set-shifting function abilities measured with Wisconsin Card Sorting Test were lower only in patients affected by both BN and previous AN, thus suggesting a predominant role of life-time AN on cognitive alterations in ED (68), rather any ED or rather than BN in particular.

Given the aforementioned body of evidence, and given the fact that cognitive functions has been interestingly suggested as a phenotype with a strong enetic background in ED (69), several authors have also investigated the role of 5HTTLPR in neuropsychological performance. Among others data from our group in University of Padua(66) have also shown that 5HTTLPR polymorphism impacts on decision making. In particular, our data showed that patients with low functioning genotypes had a poorer performance at Cognitive Bias Task, revealing an external context-independent perseverant decisional process in these subjects.

4.2 METHODS

Participants and genotyping. We included 227 patients with AN and 198 HCs. 5HTTLPR polymorphism genotyping has been previously described. We collected information about the minimum life-time BMI. All patients were affected by ED, according to DSM-IV (32).

Neuropsychological assessment. Patients were administered with the following tests. Edinburgh Handedness Inventory (a scale to measure the dominance of a person's right or left hand in everyday activities) (70), Wisconsin Card Sorting Test (a test where participants are told to match the cards, are not told how to match them, but are told if the matching is right or wrong, according to rules that continue to change - it determines set-shifting abilities), Trail A making test, Trail B making test (for visuo-spatial abilities and cognitive flexibility – in trail making A the subject is required to connect a set of 25 numbered dots (1,2,3,4…) as quickly and accurately as possible, in trail making B the subject has to alternate numbers and letters (1,A,2,B,3,C…)) (71), Iowa Gambling Task (attempts to simulate real-life decision making offering the participant 4 card sets, each associated with different money gain in the long run; the subject is asked to win as much money as possible), Cognitive Bias Task (measures how the decision-making is influenced by internal or external information, and how perseverative it is).

Statistical analyses. We used the t-test for independent samples to determine if performance at the above mentioned neuropsychological functions differed between patients with low functioning 5HTTLPR S allele and patients without it.

4.3 RESULTS

Participants. Among patients, 173 had the low functioning genotype, and 54 had the high functioning one. Minimum life-time BMI was significantly lower in patients with 5HTTLPR S allele compared to those with high functioning genotype ($p=0.007$)

Trail making test. Trail B making test and the difference between Trail A and trail B making test performance were significantly impaired in patients with S allele ($p=0.009$, $p=0.046$).

Cognitive Bias task. Patients with S allele had a significantly worse performance in CBT, compared with patients with high functioning genotype. These results are consistent with our results from a smaller population, previously published (66).

Iowa Gambling Task, Wisconsin Card Sorting test, Edinburgh Handedness Inventory. No significant difference was found according to 5HTTLPR polymorphism.

4.4 DISCUSSION

Our results show that 5HTTLPR polymorphism plays a role in neuropsychological functions in patients with AN, with S allele determining a worse performance in environment dependent decision making process, and worse cognitive flexibility. Also, our results show how having 5HTTLPR S allele is associated with a life-time lower BMI.

Our positive results regarding cognitive bias task could suggest that a more internally based decisional process, less linked to external contexts, thus to reality, could play a role in ED pathogenesis and/or maintenance. Patients affected by both AN and BN often do not seek for help in fact, and in some severe cases refuse treatment, despite severe comorbid organic life-threatening complications. It could be argued that even if they understand medical information about the eventually dramatic prognosis of their condition, they are not allowed to decide according with external inputs, due to the internally driven perseverative decisional process underpinning their behavior. Thus, due to this perseverative error and defect of adaptive choices given a set of factual environmental information, it is possible that patients only engage in any treatment only once more severe status is reached, included a lower BMI or organic complications.

Moreover, S allele moderating Trail making B test could suggest that patients with the low functioning polymorphism may have a lower ability to "reset" or change the extremely strict thoughts that influence their daily behaviors, and may experience far more difficulties in interrupting the ED cognitive and behavioral loop that mantains the disease.

We also described no influence of 5HTTLPR alleles in set shifting and decision making abilities; while S allele has been described mediating worse decision making in suicide attempters (72) and cannabis users(73), it may be argued that a peculiar disease specific interaction between genes and cognitive functions could result in different phenotypes in different diseases. Moreover, differently from all other psychiatric disorders, Ed and AN in particular are associated with clinically relevant weight alterations. Hence it could be also argued that a lower BMI in turn moderates neuropsychological impairment, and that its influence may silent or overcome smaller effects due to toher factors such as genetic polymorphims; however only longitudinally designed studies could clarify this eventually bidirectional relation.

These data and others from our group (66)furtherly suggest that 5HTTLPR polymorphism, even if not involved as causal factor per se(28), could play a role in the complex multifactorial maintaining loop of ED. Having a life-time AN diagnosis was associated with poor Cognitive Bias Task, which we suggest could increase the risk for less resilient behaviors and ED relapse. We believe that

5HTTLPR genotyping could help clinicians in detecting those subjects with poorly adaptive decision making since the very first assessment, focusing more on decisional processing rather than other psychopathologic aspects, which are hardly susceptible of any improvement due to rigidity and perseverative internally driven behaviors which could maintain EDs, regardless the diagnostic subtype.

5. BRAIN CONNECTIVITY, 5HTTLPR AND ANOREXIA NERVOSA

5.1 INTRODUCTION

Many factors have been associated to anorexia nervosa (AN) pathogenesis, and among these structural brain alterations as well. A large body of literaure has been published on functional (74) and structural(75) neuroimaging in AN. Several studies have shown that during acute phase of AN, both grey matter (GM) and white matter (WM) are decreased in volume, but that this volumetric reduction encounters a "restitutio ad integrum" after weight recovery. However too few longitudinal studies included healthy patients suffering from Anorexia Nervosa afterwards, thus it's still debated whether structural brain alterations in AN are only linked to malnutrition, dehydration or weight loss, or if they occur before and determine the weight loss itself. Actually, only few structural studies considered comorbid diagnoses which could decrease specific brain areas, medications, hydratation (76), and total intracranial volume; a recent meta-analysis has defined results about structural alteration in AN as "inconclusive" (77). More solid conclusions have been drawn about functional magnetic resonance in AN, with emotion and food processing related neural pathways showing some consistent degree of alteration across studies, in particular in amygdala (74).

Brain connectivity can be considered one of the most complex systems in nature. But, differently from other systems in nature, brain has a specific structure, with each anatomical region supposedly linked to a specific function occupying a precise position. Due to the fact that systems in nature often do not behave according to specific spatial borders (78) thus increasing a system's complexity, the brain could reveal as a favorable field to apply complex network model analysis. Briefly, a network can be defined as a mathematical representation of a real world complex system (79), defined by a collection of nodes and links between pair of nodes. Links can be thought as connections between different brain areas, in a large scale approach. They can be either binary/anatomical (white matter tracts), functional (temporally related activities in different brain regions) (80), or effective (direct or indirect causal relation between two areas that may be estimated from observed perturbations)(81). If we apply this model to the brain, the choice of nodes should ideally be well motivated on the base of solid anatomical or functional a priori criteria, should include the entire brain, and should not divide it into overlapped regions (82). On the other hand, while binary anatomical connections are either present or absent, functional and effective connectivity always need to be filtered via a weighted threshold, necessary to remove noise and other unrelated signals (83). Roughly, nodes and connections can be studied through three main sets of measure: functional segregation, functional integration, and small-world brain connectivity. Functional segregation can be defined as the ability for specialized processing to occur within densely interconnected groups of brain regions (79). Local

efficiency (measure of a network's tendency to create subgroups of strongly interconnected elements) and modularity (estimates of how a network includes many links connecting nodes within a module, and few links among modules) are two indexes of segregation. Global efficiency (topological estimate of distance between graph's elements) is a measure of integration. Sigma or "small-worldness" is the ratio between mean normalized clustering coefficient and mean normalized path, and stands for the efficiency at which information can be processed.

The aim of this study is to test the effect of 5HTTLPR polymorphism on MR acquired brain connectivity data, in consideration of neuro-cognitive altered functions in AN (see above, chapter 4). In this context we are also creating a multimodal database containing neuroimaging, neuropsychological, biological, and genetic data from Bio.Ve.D.A.

5.2 METHODS

Participants and genotyping. A sample of 74 subjects (38 healthy controls (HC), 36 AN) were scanned with 1.5 Tesla MR. 5HTTLPR polymorphism genotyping has been previously described. Low functioning genotypes were the majority in both HCs (26 out of 38 participants) and in patients with AN (29 out of 36 participants).

Magnetic resonance. A 1.5 Tesla MR tool was used. The following sequences were acquired: 1) T1 brain scan for high resolution structural brain images , 2) T2/FLAIR to detect eventual structural lesions, 3) BOLD sequences to analyze functional connections among brain areas.

Network construction: The Graph Analysis Toolbox (GAT) was used to compute network analysis. We parcellate the brain in 128 cortical and subcortical non-overlapping areas with freesurfer in order define the network nodes. The coordinates for these regions were determined using the Destrieux Atlas. To construct a connectivity matrix for each subject, we extracted the regional mean time series for each node and calculated Pearson correlations between all pairs. Correlations on the diagonal of the connectivity matrix were set to zero. In order to avoid the possible confounding that may result from the application of an absolute threshold, we applied a range of proportional thresholds to each individual association matrix to compare the network topologies across that range. Age and Edinburgh Handedness Inventory scores are used as covariates.

Network measures: Segregation properties of the network are measured with modularity coefficient and local efficiency. Modularity is evaluate by subdividing the network into groups of regions that have maximal within group connections and minimal between-group links. The quantification of modular structure is optimized with GN algorithm. Local efficiency of a node is the inverse of the

average shortest path connecting all neighbors of that node and represent a measure of the clustering properties of the network. Integration properties of the network are measured with global efficiency, computed as the average inverse shortest path length in the network. The Small World property of the network is computed as the ratio between clustering coefficient and the characteristic path length, normalized to the corresponding values of a random graph with the same number of nodes, links and degree of the nodes.

5.3 RESULTS

Participants characteristics. Mean age of HC was 25.3±6.3 years old, and mean BMI was 19.6±1.6 Kg/m^2. Mean age of patients with AN was 26.1±7.2years old, and mean BMI was 15.8±1.8 Kg/m^2.

Integration measures. No difference was found about global efficiency in both HC and patients with AN according to 5HTTLPR polymorphism.

Segregation measures. S allele tended to a significant association with higher modularity (p=0.07), and local efficiency (=0.11) compared with high functioning genotype in HC. On the contrary in patients with AN, S allele tended to confer a lower modularity (p=0.1) and lower local efficiency (p=0.1) compared with high functioning genotypes (fig 5, 6, 7).

Small-worldness measures. S allele tended to a significant association with higher sigma (small world network) (p=0.051), compared with high functioning genotypes. On the contrary in patients with AN, S allele conferred a lower sigma (p=0.003) compared with high functioning genotype (fig 8).

5.4 DISCUSSION

Our results suggest that S allele confers a lower segregation in connectivity in patients with AN with a low functioning genotype, while a higher segregation in HCs on the contrary. Despite preliminary nature of our data, and the poor available literature focusing on graph theory applied to brain connectivity of AN, several considerations should be made when discussing our results. First of all it must be mentioned that despite the small sample size, here we replicate findings from Servaas (84) and the Groningen group of Neuroimaging group in collaboration with Cambridge Cognition and Brain Sciences Unit, which recruited 120 women and found a higher segregation in subjects carrying the 5HTTLPR S allele. Authors suggest that in healthy subjects with S allele, poorer cognitive control subnetworks connections with other brain areas could explain attentional bias (85) and increased emotional reactivity (86), which in turn could explain some of the associations between 5HTTLPR low functioning genotype and several psychiatric disorders listed in the introduction of this final dissertation, such as borderline personality disorders, and antisocial behavior.

However our observations seem to suggest a different role of the 5HTTLPR genotype in mediating the functional brain network architecture between AN patients and healthy controls. Such a difference is of great interest considering the role of serotonin transporter in the regulation of emotional and behavioural control processes, in executive functioning and in the modulation of cortico-limbic circuits. Serotoninergic pathways are indeed crucial for the coupling between cognitive and emotional processes and therefore, on the basis of our data, we can suppose that the hypothesized impaired balance between bottom up and top down circuits that characterize the neurobiology of AN could be partially moderated by 5HTTLPRpolymorphism. However the cross-sectional nature of our study doesn't allow to identify if the 5HTTLPR genotype act more on the risk of developing the disorder or if it exerts a role in combination with the acute features of AN instead though (see limits section).

Beyond AN, while both integration and segregation are necessary for the optimal functioning of a small-world network (87), modularity has been linked to a better response to cognitive training after brain injury (88), but to higher impulsivity(89) as well. Moreover while in autism spectrum disorder the S allele is associated with poorer default mode network connectivity, thus higher segregation, and in turn poorer social functioning in children and adolescents(90), higher modularity has been associated with better working memory (91) as well. While such information need to be replicated in further studies, and more importantly do not match our population of interest (AN), they provide an example of how the same connectivity alterations can yield positive or negative clinical implications, depending on the involved areas, the studied outcome, and the population of interest. Alongside with the whole brain analysis, more specific function-related networks should be a priori set as the object of interest to investigate integration and segregation in specific neuropsychological alterations or psychiatric disorders. While ideally integration of separate information coming from highly effective modules has been described as typical of HC compared with subjects affected by other psychiatric disorders(92, 93), proper segregation might be necessary for specific neuropsychological tasks beyond the whole brain degree of integration (91). Thus the lower segregation of the global connectivity network in AN patients with low functioning genotype is interesting since these patients show impaired executive functioning and a life time lower BMI. If modular network architecture provides greater resilience capacities if compared to more integrative functional patterns, and is more economic from an energetic and a metabolic point of view, we can hypothesize that the capacity to modulate the functional network in a more clustered fashion can be really important in a disorder with high energy-saving needs in order to advantageoulsy adapt the emotional, cognitive and behavioral responses to the environmental needs. The failure of S carriers patients in operating an effective modular reorganization of the network in the presence of starvation and malnutrition could underpin a disfunctional unbalance between emotional and cognitive control processing that can lead

to difficulties in adaptive responses selection processes. We can assume that these compensatory difficulties can partially explain the greater inflexibility that characterizes those patients who show more resistances to conventional treatment plans and who might be candidates for more individualized treatment strategies. Thus the lower network segregation in S carriers may be relative to a reduced compensatory capacity of the functional network in underweight conditions, with relative worsening of those performances for which a discrete and modular operations is necessary.

Beyond the relevance of our findings in the long process of identification of biological mechanisms underlying clinical phenotypes in AN, our results may open the way for a deeper insight into the possible role of brain network analysis in the early screening for AN, or even in the identification of the at risk subjects. Similarly to what has been suggested in Alzheimer's disease(94) where peculiar brain connectivity is described accordingly to the graph theory, magnetic resonance scanning could be used, in association with 5HTTLPR polymorphism for diagnostic or prognostic aims in the future. Moreover in both Alzheimer's disease (95) and schizophrenia (96) brain connectivity as measured accordingly to graph theory, results impaired since pre-clinical or ealry stages of the disease. However only studies with longitudinal designs will show whether the results we are showing will extend to at risk subjects, or in other words whether they are a trait disease pre-existing condition or a state under-weight immanent alteration.

The present study has two main points of strength. First of all it is one of the first studies which investigates brain connectivity according to the graph theory in AN. Secondly, it also investigates the role of 5HTTLPR polymorphism, following a multimodal research approach which provides far more information beyond each single research approach per se.

However several limitations should be mentioned as well. The main limitation is the small sample size, which is due to the pilot nature of the study and its preliminary phase. This limited the statistical significance of our results, which however had a solid and consistent trend towards an effect of 5HTTLPR on brain connectivity in AN. Secondly, patients were scanned with 1.5 Tesla MR device, while 3Tesla would have provided data from higher resolution scans. However 1.5 Tesla devices are still widely used in research. Third, the study had a cross-sectional design; thus any causal or prognostic consideration was not allowed. Fourth, we only included patients with AN, excluding those affected by BN. However due to pilot nature of our investigation we chose to focus on the diagnostic group where more solid evidence was available about neuropsychological functioning, and this was the AN case. Fifth, we here report the whole brain analysis results; however we also ran the subnetwork analyses which however did not yield any significant result.

In conclusion, even if 5HTTLPR does not seem to play a role in AN onset (28), it does play a role in its specific neuropsychological functions(66), and this study suggests a neural connectivity substrate underpinning such alterations.

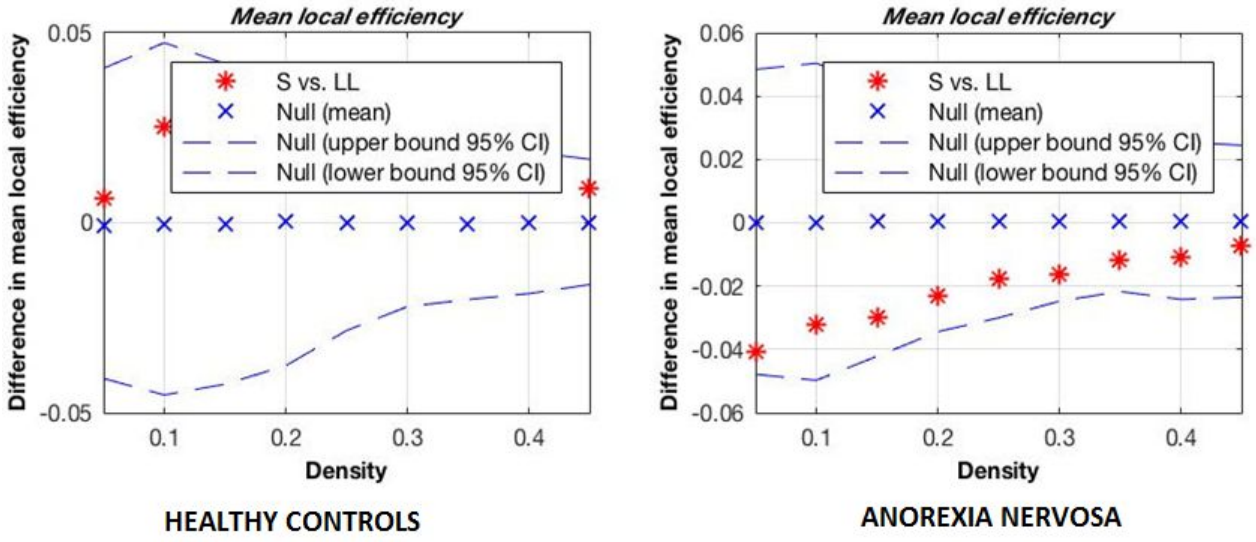

Fig 5. Mean Local Efficiency and 5HTTLPR polymorphism in healthy controls and anorexia nervosa.

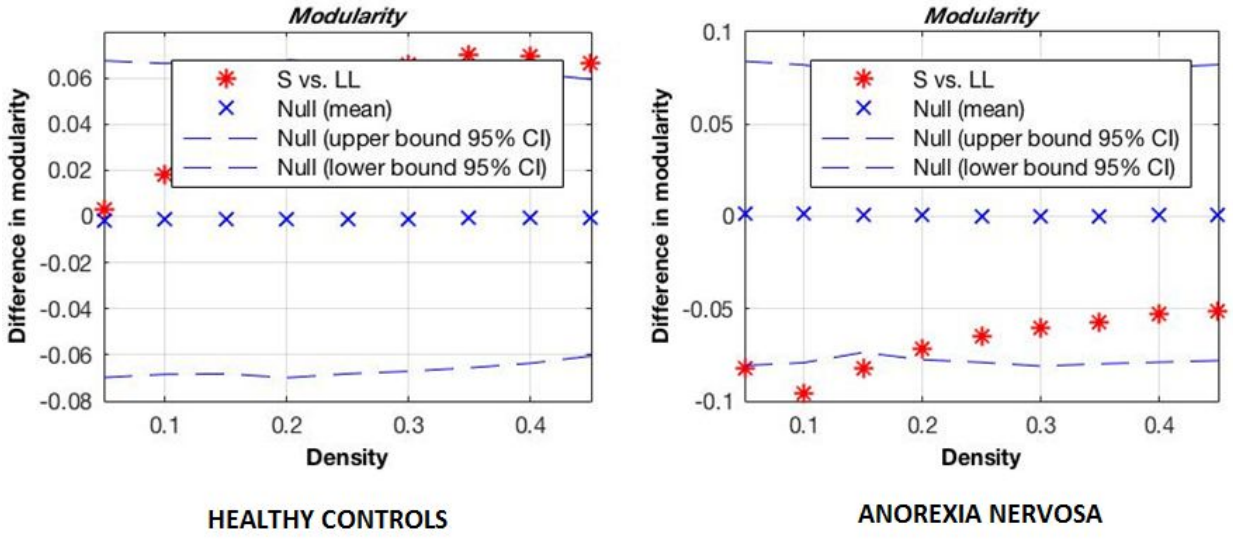

Fig 6. Modularity and 5HTTLPR polymorphism in healthy controls and anorexia nervosa.

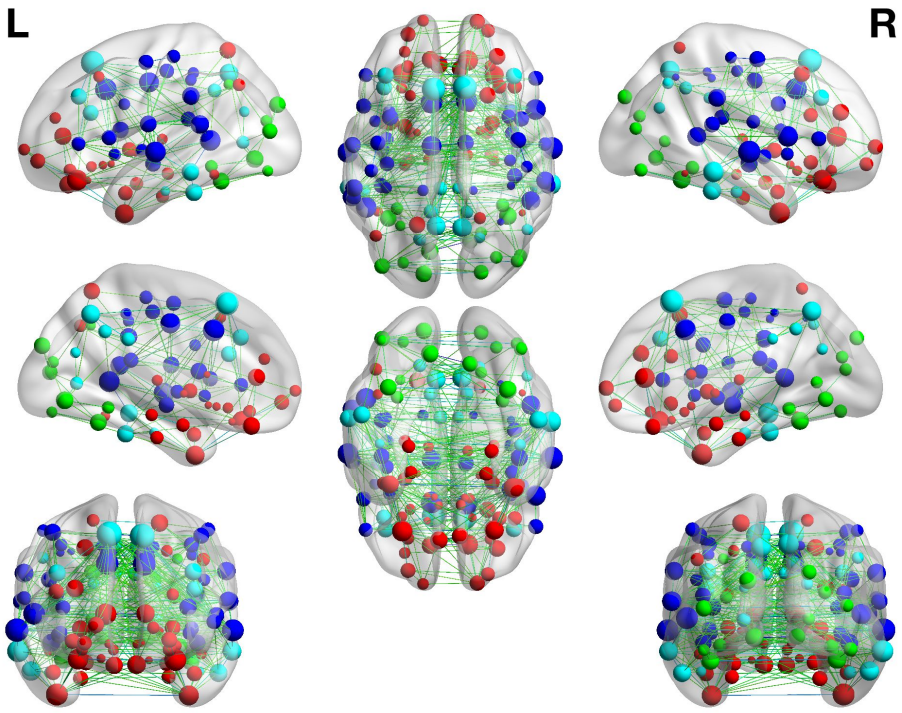

Fig 7. Brain connectivity in patients with anorexia nervosa and 5HTTLPR low functioning genotype.

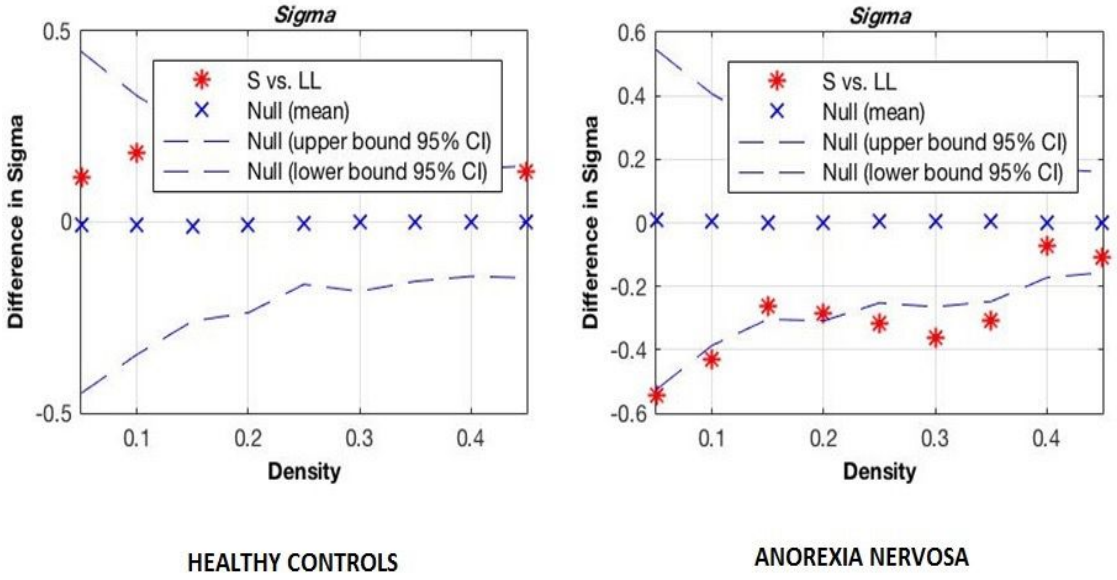

Fig 8. Sigma "small-worldness" and 5HTTLPR polymorphism in healthy controls and anorexia nervosa.

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