The role of genetic variability in the SLC6A4, BDNF and GABRA6 genes in anxiety-related traits

Arias B, Aguilera M, Moya J, Sáiz PA, Villa H, Ibáñez MI, García-Portillo MP, Bobes J, Ortet G, Fañánás L. The role of genetic variability in the SLC6A4, BDNF and GABRA6 genes in anxiety-related traits.

Objective: The aims of this study were to test the individual association of the serotonin transporter gene (SLC6A4), the brain-derived neurotrophic factor gene (BDNF) and the GABA receptor subunit gene (GABRA6) with anxiety-related traits and to explore putative gene–gene interactions in a Spanish healthy sample.

Method: A sample of 937 individuals from the general population completed the Temperament and Character Inventory questionnaire to explore Harm Avoidance (HA) dimension; a subsample of 553 individuals also filled in the Big Five Questionnaire to explore the Neuroticism dimension. The whole sample was genotyped for the 5-HTTLPR polymorphism (SLC6A4 gene), the Val66Met polymorphism (BDNF gene) and the T1521C polymorphism (GABRA6 gene).

Results: Homozygous individuals for the T allele of the T1521C polymorphism presented slightly higher scores for HA than C allele carriers ($F = 2.96, P = 0.019$). In addition, there was a significant gene–gene interaction on HA between the 5-HTTLPR and Val66Met polymorphisms ($F = 3.4, P = 0.009$).

Conclusion: GABRA6 emerges as a candidate gene involved in the variability of HA. The effect of a significant gene–gene interaction between the SLC6A4 and BDNF genes on HA could explain part of the genetic basis underlying anxiety-related traits.

Significant outcomes

- Part of the interindividual differences observed for anxiety traits measured by the Harm Avoidance dimension could be explained by i) the GABRA6 gene variability and ii) a complex gene–gene interaction between the SLC6A4 and brain-derived neurotrophic factor gene.
- The Harm Avoidance dimension of the Temperament and Character Inventory questionnaire is more strongly supported by genetic components than the Neuroticism dimension of the Big Five Questionnaire.

Limitations

- The two Spanish populations analysed presented differences with respect to the genotype distribution of the Val66Met polymorphism (brain-derived neurotrophic factor gene).
- Neuroticism data from the Big Five Questionnaire were only available for 57% of the total sample of general population.
Complex genetic effects on anxiety-related traits

Introduction

Anxiety-related traits have described as continuously distributed in the normal human personality (1). They have been defined as individual differences in emotional reactivity, proneness to worry and susceptibility to negative affect. These anxiety-related traits are mainly captured by the presence of Neuroticism (2, 3) or Harm Avoidance (HA) (4). Neuroticism (N) is a psychological dimension measured by NEO personality inventory (2, 3) and includes traits such as anxiety, anger, hostility, depression, self-consciousness, impulsiveness and vulnerability. On the other hand, HA dimension, that is captured by the Temperament and Character Inventory (TCI) (4), characterizes individuals with high scores for being cautious, tense, apprehensive, fearful, inhibited, shy, easily fatigued and worried. Both Neuroticism and HA have been considered as markers for vulnerability to affective disorders (5–9). For instance, it has been shown that Neuroticism reflects the genetic vulnerability to major depression, sharing estimated 50% of the genetic liability (9–11).

Twin studies have suggested that the estimated heritability for personality traits ranges between 30% and 50% (12). Specifically, these studies on the genetics of personality have demonstrated that the genetic component of anxiety-related traits accounts for 40–60% of the observed variance (13, 14). Then, anxiety-related traits could be considered as a complex trait in which multiple genes of small effect are playing a role together with environmental factors [see reviews of (15–17)].

There is growing interest about the relation between neurobiological functions and normal variations in personality traits (18–20). In this sense, serotonin neurotransmission has a fundamental role in both the modulation of emotional behaviour and the brain development. Genetic variability associated with serotonergic function is likely to influence behavioural predispositions such as anxiety-related traits and depressive traits (21–26). The serotonin transporter (5-HTT) has a key role in this system, because it is the main reuptake mechanism and the main biological target for antidepressant drugs. The 5-HTT is encoded by a single gene (SLC6A4) on chromosome 17q11.1–17q12. A common polymorphism has been described in this gene; a 44bp insertion/deletion variant (5-HTTLPR) placed on the promoter region. The short variant of this polymorphism (S allele) reduces the transcriptional efficiency of the gene, resulting in decreased serotonin transporter expression in the neuron (27, 28).

With respect to this polymorphism, the seminal study carried out by Lesch et al. (28) showed an association between anxiety-related traits and the SLC6A4 gene. Although inconsistencies have emerged [see review (15, 29)], one recent meta-analysis has shown the effect of variability at SLC6A4 gene on depression and stress sensitivity (30).

Besides the 5-HTT gene, the brain-derived neurotrophic factor (BDNF) from the neurotrophin family could be of special interest because of its critical role in normal adaptive responses to stress as well as in the response to antidepressant treatment (31, 32). The BDNF gene is located in chromosome 11p14 and presents a single-nucleotide polymorphism (SNP) (196 G>A) that provokes a change of Valine to Metionine at position 66 of the protein sequence with functional consequences (33). The Val variant is associated with higher neuronal BDNF secretory activity than is the Met variant (34). The secretion of BDNF is crucial for the growth and differentiation of developing neurons in both central and peripheral nervous systems, and BDNF is also implicated in the survival of neuronal cells in response to stress (34, 35). Sen et al. (36) reported that the Met allele of the Val66Met polymorphism at the BDNF gene was associated with lower levels of Neuroticism. Recently, this Met allele has been shown to be associated with anxiety disorders that is, individuals carrying the Met allele showed impaired learning of cues signalling safety vs. threat that rely on extinction mechanisms (37). However, several studies failed to replicate such findings (38–40), including a recent meta-analysis exploring the effect of this polymorphism on HA and Neuroticism (41).

Another neurotransmitter that might be involved in the propensity for a fearful or anxious temperament is γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the CNS (42, 43). The inhibitory effect of GABA is mediated by GABA_A receptors. This receptor is one of the targets of anxiolytics and benzodiazepines drugs that act as agonists of these receptors increasing the efficiency of the inhibitory GABAergic neurotransmission.

It has been suggested that individual differences in the frontal cortical GABA_A receptor complex and GABA systems could modulate patterns of brain activity associated with individual differences in threat-related responses (42). Uhart et al. (44) analysed a single-nucleotide polymorphism (SNP) consisting of a T-to-C substitution (position 1521) in the 3’ non-coding region of the GABA_A2Cx receptor subunit gene GABRA6 (cr. 5q34) in a healthy
population. The results revealed that homozygotes for the C allele showed an attenuated hormonal and physiological response to acute psychological stress. Therefore, this polymorphism could be involved in the individual differences underlying part of the biological basis of anxiety-related traits.

Aims of the study
The aims of this study were i) to test the individual association of the SLC6A4 (5-HTTLPR polymorphism), the BDNF gene (Val66Met polymorphism) and the GABRA6 gene (T1521C polymorphism) with anxiety-related traits (Neuroticism and HA) in a sample of a healthy Spanish population; and ii) to explore the putative gene–gene interactive influences of these polymorphisms on anxiety-related traits.

Material and methods
Sample
Our sample consisted of 937 subjects from the general population (47.8% males; total mean age = 30.5, SD = 12.2) who were recruited from the campus of the University Jaume I (Castellón, Spain) and from primary care settings from Oviedo (Asturias, Spain).

In terms of education, 12.2% of individuals had completed elementary school, 56.8% had completed high school and 25% had received a university education. Sociodemographic data divided by the geographical origin of the samples (Castellón/Asturias) are displayed in Table 1.

Exclusion criteria were the presence of any past or present major psychiatric disorder and/or a history of any severe mental disorder in first-degree relatives. In the sample from Castellón, these aspects were screened by means of a short interview designed ad hoc for this study on the basis of selected items of structured scales such as SCID-I (45) and FIGS (46). Specific questions about psychiatric assistance, psychotropic medication, hospital admissions and suicide attempts were asked to the participants. In the Oviedo sample, these aspects were screened using the Spanish version of the Mini-International Neuropsychiatric Interview (MINI DSM-IV criteria) (47).

All participants were of Spanish (Caucasian) ancestry (based on the birthplace of their four grandparents) to reduce the possibility of confounding genetic differences by population stratification (48).

Ethical approval was obtained from local Spanish research ethic committees in each Institution. All participants provided written informed consent before inclusion in the study.

Measurements
Personality assessment. All participants filled out the self-reported TCI (49) composed of 240 items. Given the aims of this study, the analyses were focused on the dimension of HA, which has four subscales: Anticipatory worry (HA1), Fear of uncertainty (HA2), Shyness with strangers (HA3) and Fatigability (HA4). HA1 referred to pessimistic worries, tendency to anticipate harm and failure and rumination about embarrassing experiences; HA2 referred to feelings of tension and anxiety in front of uncertainty or unfamiliar circumstances that are potentially dangerous; HA3 referred to shyness and unassertively behaviour in most social situations; and HA4 include aspects of fatigability and low energy. Both the original TCI (49) and the Spanish version have shown adequate psychometric properties (50, 51).

In addition, participants from the sample of Castellón (n = 533) completed the self-report Big Five Questionnaire [BFQ; (52)]. The BFQ presents 44 items that will define the Big Five domains (53). The analyses have been focused on the dimension of Neuroticism (N) that includes eight items about the tendency of being worried, tense, nervous or depressed. However, these subscales were not available in our sample. The Spanish BFI scales have shown high internal consistency, retest reliability and clear factor structure, as well as strong convergence with longer Big Five measures (52).

Laboratory methods. Genomic DNA from the Castellón subsample was extracted from saliva samples using the Collection Kit BuccalAmp DNA extraction kit (Epicentre® Biotechnologies, Madison, WI, USA). Genomic DNA from the Oviedo subsample was extracted from blood samples using the salting-out technique (54). The 5-HTTLPR polymorphism of the serotonin transporter gene was analysed using the protocol.
previously described by Lesch et al. (28). The SNP rs6265 (Val66Met) of the BDNF gene and the SNP rs3219151 (T1521C) of the GABRA6 gene were genotyped using Applied Biosystems (AB) TaqMan technology. An AB assay-on-demand service supplied the probes.

Table 2 shows the final genetic sample. Of the total sample, the 93.8% was successfully genotyped for the 5-HTTLPR-SLC6A4 gene, the 91.2% for the Val66Met-BDNF gene and the 91% for the T1521C-GABRA6.

We randomly select a 10% of the individuals to re-genotype them to confirm the reproducibility of the pattern. The reproducibility was 100%.

Statistical analyses

Analyses were performed using Stata 9.1 (55), EpiInfo (56) and G*Power (57).

All regression analyses were controlled for age, gender and demographic origin.

The main effects of polymorphisms (5HTTLPR-SLC6A4 gene, Val66Met-BDNF gene and T1512C-GABRA6 gene) on HA and Neuroticism were analysed separately using linear regression analysis. Regressions were performed for the HA dimension and separately for each individual subscale of the dimension. The Wald test was performed to test the overall main effect of each polymorphism on each dimension and subscale.

The xi3 command was then used to test interactive effects with categorical predictors (58). The interactive effects between SLC6A4 5-HTTLPR and BDNF Val66Met, between SLC6A4 5-HTTLPR and GABRA6, and between BDNF Val66Met and GABRA6 T1512C were fitted in models of HA and Neuroticism. The Wald test was used to assess the interaction effect. When a two-way interaction was significant, further simple effects were assessed, that is, the effect of VI1 at each level of VI2.

G*Power v3 was used to calculate the statistical power; in our sample, we had a sufficient power of 0.80 to detect small effect sizes in the study ($w = 0.11$).

Table 2. Genotype distribution of the three polymorphisms analysed according to geographical origin

| 5-HTTLPR polymorphism (SLC6A4 gene) | Allele distribution (%) |
|-------------------------------------|-------------------------|
| L/L 241 (27.4) L/S 422 (48) S/S 216 (24.6) | L allele 904 (51.4) S allele 854 (48.6) |
| Castelló (n = 475) 119 (25.1) 230 (48.4) 126 (26.5) | Castelló (n = 475) 468 (49.2) 482 (50.8) |
| Oviedo (n = 404) 122 (30.2) 192 (47.5) 90 (22.3) | Oviedo (n = 404) 436 (53.9) 372 (46.1) |
| $\chi^2 = 3.75$, df = 2, $P = 0.15$ | $\chi^2 = 3.86$, df = 1, $P = 0.05$ |

| Val66Met polymorphism (BDNF gene) | Allele distribution (%) |
|-----------------------------------|-------------------------|
| Val/Val 551 (64.4) Val/Met 265 (31) Met/Met 39 (4.6) | Val allele 1367 (79.9) Met allele 343 (20.1) |
| Castelló (n = 470) 282 (60) 159 (33.8) 29 (6.2) | Castelló (n = 470) 723 (76.9) 217 (23.1) |
| Oviedo (n = 385) 269 (69.9) 106 (27.5) 10 (2.6) | Oviedo (n = 385) 644 (83.6) 126 (16.4) |
| $\chi^2 = 11.83$, df = 2, $P < 0.01$ | $\chi^2 = 11.93$, df = 1, $P < 0.01$ |

| T1512C polymorphism (GABRA6 gene) | Allele distribution (%) |
|-----------------------------------|-------------------------|
| T/T 281 (33) T/C 429 (50.4) C/C 141 (16.6) | T allele 991 (68.2) C allele 711 (41.8) |
| Castelló (n = 481) 161 (33.5) 236 (49.1) 84 (17.5) | Castelló (n = 481) 558 (68.1) 404 (41.9) |
| Oviedo (n = 370) 120 (32.4) 193 (52.2) 57 (15.4) | Oviedo (n = 370) 433 (68.5) 307 (41.5) |
| $\chi^2 = 1$, df = 2, $P = 0.61$ | $\chi^2 = 0.04$, df = 1, $P = 0.83$ |

*Frequencies were in Hardy–Weinberg equilibrium for the Castelló subsample (5-HTTLPR polymorphism $\chi^2 = 0.24$, $P = 0.88$; Val66Met polymorphism $\chi^2 = 0.52$, $P = 0.77$; T1512C polymorphism $\chi^2 = 0.02$, $P = 0.98$; the Oviedo subsample (5-HTTLPR polymorphism $\chi^2 = 0.36$, $P = 0.83$; Val66Met polymorphism $\chi^2 = 0.00$, $P = 0.99$; T1512C polymorphism $\chi^2 = 1.06$, $P = 0.59$); and for the total sample (5-HTTLPR polymorphism $\chi^2 = 0.70$, $P = 0.70$; Val66Met polymorphism $\chi^2 = 0.51$, $P = 0.76$; T1512C polymorphism $\chi^2 = 0.60$, $P = 0.74$).

**Statistical results derived from the comparison between the Castelló and Oviedo subsample distributions.

BDNF, brain-derived neurotrophic factor.
Results

The genotype, allele distribution and Hardy–Weinberg equilibrium of each population (Castelló and Oviedo) as well as those of the total sample are shown in Table 2.

The genotypic frequencies that we observed in these Spanish populations were similar to those frequencies described for European Caucasians (39, 44, 59, 60).

The two populations presented similar genotype and allele distributions for the 5-HTTLPR polymorphism (SLC6A4 gene) and for the T1512C polymorphism (GABRA6 gene). However, the genotype and allele distribution differed between Castelló and Oviedo for the Val66Met polymorphism (BDNF gene) (genotype frequencies: \( \chi^2 = 11.83, df = 2, P = 0.003 \); allele frequencies: \( \chi^2 = 11.93, df = 1, P < 0.001 \)). These differences were taken into account when we merged the samples for subsequent statistical analyses.

Main effects of the analysed genes on anxiety traits

Main outcome measures. We found no significant associations between the 5-HTTLPR (SLC6A4 gene) or Val66Met (BDNF gene) polymorphisms and HA.

Interestingly, the effect of GABRA6-T1512C genotypes on HA approached statistical significance (\( F = 2.96, P = 0.051 \)). When we compared C allele carriers (TC or CC) vs. TT homozygous subjects for HA scores, we found that C-carriers scored significantly higher than TT subjects (\( F = 5.49, P = 0.019 \)).

We found no association between either of the polymorphisms analysed on Neuroticism.

Secondary outcome measures (subdimensions of HA). We did not find any significant associations between the 5-HTTLPR (SLC6A4 gene) or Val66Met (BDNF gene) polymorphisms and HA subdimensions.

Interestingly, a significant effect of the GABRA6 T1512C polymorphism was found on the subdimension of anticipatory worry (\( F = 4.07; P = 0.017 \)). Specifically, the TT homozygous group had significantly higher scores compared with C-carriers subjects (TC or CC) (\( F = 8.08, P = 0.005 \)). No significant effect was found on the other subdimensions (see Table 3).

Gene and anxiety-related trait interactions

We found a significant two-way interaction in relation to HA. An interaction between the 5-HTTLPR (SLC6A4 gene) and Val66Met (BDNF gene) polymorphisms modulated the HA dimension scores (\( F = 3.4, P = 0.009 \)). Analysis of simple effects showed that subjects with Met/Met and S/S genotypes had higher scores on HA compared with Met/Met-LS or Met/Met-L (\( F = 3.43, P = 0.033 \) (see Fig. 1).

No other gene–gene interactions were significant with regard to HA or Neuroticism.

Discussion

In reference to the main effect of the 5-HTTLPR polymorphism (SLC6A4 gene) on anxiety-related traits, we did not replicate the previously association reported by Lesch et al. (28). In contrast, our results agree with most of the studies that have reported negative results in Caucasian populations (61–68). In this sense, a recent meta-analysis has also shown this lack of association between the 5-HTTLPR polymorphism of the SLC6A4 gene and HA or Neuroticism (29).

It has been suggested that important sources of heterogeneity between studies could be due to the

| Table 3. Mean scores (SD) and results (Wald test) per polymorphism analysed in the whole sample for Harm Avoidance (HA) and its subscales (\( n = 937 \)) |
|---------------------------------------------------------------|
|                               | 5-HTTLPR polymorphism | BDNF Val66Met polymorphism | GABRA6 T1512C polymorphism |
|                               | LL (n = 214) | LS (n = 422) | SS (n = 216) | F | Val/Val (n = 551) | Val/Met (n = 265) | Met/Met (n = 39) | F | TT (n = 281) | TC (n = 429) | CC (n = 141) | F |
| HA                             | 15.6 (6.25) | 15.5 (6.06) | 15.9 (6.51) | 0.52 | 15.57 (6.09) | 15.47 (6.44) | 17.07 (7.33) | 0.13 | 16.29 (6.60) | 15.31 (6.34) | 15.21 (6.33) | 2.96* |
| Anticipatory worry subscale (HA1) | 4.24 (2.49) | 4.19 (5.59) | 4.16 (2.69) | 0.00 | 4.17 (2.57) | 4.14 (2.67) | 4.76 (2.95) | 1.38 | 4.58 (2.75) | 4.05 (2.59) | 4.07 (2.66) | 4.07** |
| Fear of uncertainty subscale (HA2) | 4.22 (1.91) | 4.16 (1.89) | 4.25 (1.89) | 0.38 | 4.22 (1.89) | 4.17 (1.91) | 4.32 (1.96) | 0.34 | 4.34 (1.99) | 4.18 (1.93) | 4.08 (1.87) | 2.10 |
| Shyness with strangers subscale (HA3) | 3.58 (2.14) | 3.55 (2.06) | 3.82 (2.00) | 1.71 | 3.59 (2.08) | 3.63 (2.10) | 4.12 (2.17) | 2.01 | 3.69 (2.05) | 3.56 (2.23) | 3.60 (2.08) | 0.39 |
| Fatigability subscale (HA4) | 3.52 (1.92) | 3.61 (1.89) | 3.89 (1.89) | 0.14 | 3.59 (1.89) | 3.54 (1.86) | 3.88 (1.79) | 0.60 | 3.69 (1.84) | 3.52 (1.96) | 3.46 (1.96) | 1.23 |

* P = 0.051; ** P = 0.017 (All analyses controlled for age, gender and geographical origin).

BDNF, brain-derived neurotrophic factor.
use of slightly different personality constructs, the inclusion of psychiatric populations, or the use of small sample sizes (17). In this study, both Neuroticism and HA were explored; in addition, the whole sample was screened for any current or lifetime psychiatry disorder and, finally, the sample size was larger than in previous studies (61–68).

The Val66Met polymorphism (BDNF gene) has previously been associated with anxiety-related traits (36, 41). These studies revealed that Val homozygous presented higher levels of Neuroticism compared with Met allele carriers. However, later studies including two recent genome-wide association studies are in agreement with our findings and did not detect this independent effect in relation to this personality trait (38–40, 69, 70).

Interestingly, a gene–gene interaction was detected between the 5-HTTLPR polymorphism (5-HTT gene) and Val66Met polymorphism (BDNF gene): among individuals with the Met/Met genotype, carriers of the SS genotype exhibited significantly higher scores for HA than L allele carriers. This gene–gene interaction may underlie the inconsistencies found in studies exploring only the main effects of one of these genes and anxiety-related traits. In this sense, a recent study published by Terracciano et al. (71) has shown this gene–gene interaction and anxiety-related traits measured by Neuroticism. Although we did not replicate the specific effect of this interaction in Neuroticism, it is interesting to note that both results shown the effect of SLC6A4 and BDNF genes on anxiety-related traits measured by personality dimensions.

In addition, the results of our study provide evidence for a putative role of the GABRA6 T1512C polymorphism in HA variability in a large sample from the general population. Specifically, we found an association between the TT genotype and higher scores on the subscale Anticipatory Worry of the HA dimension. These results together with the results reported by Uhart et al. (44) suggest that this gene, in accordance with our hypothesis, is strongly associated with individual differences in anxiety-related traits, specifically in those traits that are closely related to the anxiety spectrum, such as anticipatory worry. However, we know that this polymorphism located at 3q region does not seem to display functional relevance. As 16 subunit is included into a cluster in chromosome 5q with z1, b2 and g2 subunit genes (72), the analysed polymorphism may be in linkage disequilibrium with another site within the GABRA6 gene or these genes located in close proximity. Moreover, GABA_A receptors are classified as ionotropic receptors which are involved in fast events in the neuron, as opposite to other type of receptors (such as the serotonin transporter) that mediate long-term effects modifying the responsiveness and plasticity of the neuron (73). We could hypothesize that subtle genetic changes in this fast-response receptors such as one nucleotide like this SNP may alter the signal transmission in the neuron impacting, then, in the final anxiety-related phenotype.

Finally, our results seem to identify genetic components involved in HA, derived from Cloninger's tridimensional theory of personality (4). However, negative results were found for the Neuroticism dimension derived from the Big Five model developed by Costa and McCrae (2). These results are consistent with a recent meta-analysis reporting the existence of a small but significant genetic background for the HA dimension but not for the Neuroticism dimension (74). Taken together, these findings suggest that the HA construct seems to be more strongly associated with genetic-related traits than the Neuroticism construct. Nevertheless, further research is needed to elucidate the complex biological and genetic architecture of anxiety-related traits.

Our study has some limitations. First, we found differences between the Castellón and Oviedo populations with respect to the genotype distribution of the Val66Met polymorphism (BDNF gene); these differences were basically related to an increase in the frequency of the Val/Val genotype in the subsample from Oviedo. However, when we merged the two populations, the genotype frequencies were similar to those expected in a Caucasian population (39). Thus, it is unlikely that the significant results obtained were because of a bias in the genotype distribution. In fact, the effect of interaction between SLC6A4 and BDNF genes on HA was found with the Met allele and not with the
Val allele, as might be expected because of the overrepresentation of this allele in the Oviedo sample.

With a sample of almost a thousand individuals, our study was larger than some of the previous studies (61–68), and therefore the likelihood of Type I or Type II errors was reduced. Multiple testing corrections are likely to be excessively exclusive in the context of the present study, because the selection of the genetic polymorphisms, the sample size and the analyses performed had a directional hypothesis based on previous findings (75). However, it should be taken into account that when we consider correction for multiple testing based on the false discovery rate (76), our significant results do not survive the correction.

In conclusion, the GABRA6 T1512C polymorphism has emerged as a putative genetic factor involved in human anxiety-related trait variability, specifically HA. Moreover, the effect of a significant gene–gene interaction between the SLC6A4 gene and BDNF gene on HA could partly explain some of the inconsistencies found in previous studies and should be taken into account in the understanding of individual differences in complex constructs such as anxiety-related traits.

Acknowledgements

This study was supported by the Ministerio de Educació y Ciencia (SAF 2005-07852-C02-01), the Ministerio de Ciencia e Innovación (SAF2008-05674-C03-01), the Ministerio de Sanidad y Consumo (05-317), the Fondos FEDER and the Ministerio de Ciencia e Innovación (PSI2008-05988) and Comissionat per a Universitats i Recerca del DIUE de la Generalitat de Catalunya (2009SGR827).

Support was also received from the Spanish Ministry of Health, CIBERSAM and the Institut de Biomedicina de la Universitat de Barcelona (IBUB). M. Aguilera thanks the Departament d’Universitats, Recerca i Societat de la Informació (DURSI) de la Generalitat de Catalunya for a predoctoral grant (2004 FI 00673). Thanks to Dr Marieke Wichers for her statistical advice.

Declaration of interest

The author(s) declare that, except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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