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Association of BDNF with anorexia, bulimia and age of onset of weight loss in six European populations

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Several genes with an essential role in the regulation of eating behavior and body weight are considered candidates involved in the etiology of eating disorders (ED), but no relevant susceptibility genes with a major effect on anorexia nervosa (AN) or bulimia nervosa (BN) have been identified. Brain-derived neurotrophic factor (BDNF) has been implicated in the regulation of food intake and body weight in rodents. We previously reported a strong association of the Met66 allele of the Val66Met BDNF variant with restricting AN (ANR) and low minimum body mass index in Spanish patients. Another single nucleotide polymorphism located in the promoter region of the *BDNF* gene (−270C > T) showed lack of association with any ED phenotype. In order to replicate these findings in a larger sample, we performed a case–control study in 1142 Caucasian patients with ED consecutively recruited in six different centers from five European countries (France, Germany, Italy, Spain and UK) participating in the 'Factors in Healthy Eating' project. We have found that the Met66 variant is strongly associated to all ED subtypes (AN, ANR, binge-eating/purging AN and BN), and that the −270C BDNF variant has an effect on BN and late age at onset of weight loss. These are the first two variants associated with the pathophysiology of ED in different populations and support a role for BDNF in the susceptibility to aberrant eating behaviors.

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INTRODUCTION

Anorexia nervosa (AN) and bulimia nervosa (BN) are apparently related eating disorders (ED) affecting 8–10% of young females and are characterized by alterations in eating behavior, weight regulation and perception of body image. They are complex syndromes that most likely result from, and are sustained by, environmental, psychological and biological factors (1). ED are associated with a high level of physical, social and psychological morbidity for the individual and family, with 40–60% of patients showing persistent disability over 20 years (2).

Genetic vulnerability to ED may involve factors relating to feeding and appetite, energy metabolism, development, personality and mood. Several genes with an essential role in the regulation of eating behavior and body weight have been considered as candidate susceptibility factors for ED, but no relevant susceptibility genes with major effect on AN or BN have yet been identified unequivocally. Physiological and animal models have demonstrated that the brain-derived neurotrophic factor (BDNF) induces appetite suppression and body weight reduction through a central mechanism that may involve the serotoninergic neurotransmitter system (3–7), and support the hypothesis that alterations on this neurotrophic system and their effect on its downstream central mediators could determine eating behavior abnormalities predisposing to AN and BN. *BDNF* encodes a neurotrophic factor involved in neuronal survival and differentiation during the development of the nervous system, and also in the synaptic efficiency and neuronal plasticity (8–10). Consistent with the above findings, we previously reported a strong association between the Met66 allele of the Val66Met BDNF variant and restricting AN (ANR) and low minimum body mass index (minBMI) in Spanish patients (11).

In order to replicate these findings in a larger sample, we performed a case–control study with six independently recruited samples from five European countries (France, Germany, Italy, Spain and UK) participating in the 'Factors in Healthy Eating' project. We analyzed the Val66Met variant and another single nucleotide polymorphism (SNP) located in the promoter region of the BDNF gene (−270C>T) in ANR, binge-eating/purging AN (ANBP), BN and in some ED-related phenotypes such as minBMI, maximum body mass index (maxBMI) and age at onset of weight loss (AO).

RESULTS

We first performed a power analysis and observed a reduced statistical power in some populations due to the limited size of their control groups, and decided to perform the case–control study comparing patients from each center to the total sample of European controls, once population heterogeneity among controls was excluded ($\chi^2 = 0.69$, $P = 0.40$ for Val66Met; $\chi^2 = 0.97$, $P = 0.32$ for −270C>T). The AN sample had a power of 99% and 62%, while the BN sample showed a power of 98% and 44% for the Val66Met and the −270C/T SNPs, respectively (data not shown). Both SNPs followed a Hardy–Weinberg distribution and linkage disequilibrium between them was observed in both patients and control groups ($\chi^2 = 10.79$, $P = 0.003$; $\chi^2 = 4.86$, $P = 0.02$).

We first studied BN patients who were recruited in four different centers. Once we excluded genetic heterogeneity among patients ($\chi^2 = 0.65$, $P = 0.42$ for Val66Met; $\chi^2 = 0.34$, $P = 0.55$ for −270C>T), the combined analysis of all populations showed a strong association of BN and the Met66 allele of the Val66Met polymorphism ($P < 0.001$, OR = 1.59; Table 1). When we separated the samples by centers, an excess of Met66 carriers was detected in all groups. These differences reached statistical significance in the Spanish and in the Milan samples (Table 1). We then considered the −270C>T sequence variant and no significant differences were detected when all populations were analyzed together. However, the analysis by populations showed that the two BN groups from Florence and Germany significantly differed from controls in both genotype and allele frequencies. Nevertheless, while the Italian patients showed an excess of the −270C allele, in the German sample it was the −270T allele that was overrepresented (Table 2). After Bonferroni correction, all associations between BN and BDNF were still positive except for the Spanish sample.

We then analyzed the AN samples obtained from six participating centers. The combined analysis of all populations showed positive association of Met66 and AN ($P = 0.0008$, OR = 1.37), ANR ($P = 0.004$, OR = 1.43) and ANB ($P = 0.012$; OR = 1.29) (Table 3). Once separated by centers, five out of the six AN groups had an excess of the Met66 allele with the exception of the German sample, which showed an increased number of patients carrying the Val66 allele. The significance of these differences was highly variable depending on the AN subtype and the population analyzed. Thus, genotype and allele frequencies significantly differed from controls in the AN, ANR and ANB groups from Milan. In the Spanish and Florencian samples association was found when the ANR subtype was considered. Moreover, in the former case the overrepresentation of the Met66 allele was also significant in the AN group. On the contrary, in the French and British samples differences reached statistical significance in the ANB group. This significance was also observed when the French AN patients were analyzed as a whole. Interestingly, the German population showed an excess of the Val66 instead of the Met66 allele, but this difference was only significant when the ANB subgroup was considered. Under the most conservative multiple comparison correction, considering two polymorphisms and two AN subtypes, differences in the Val66Met variant were still significant in all populations with the exception of the Florencian and German samples. When we considered the −270C>T polymorphism no significant differences were found in any of the AN groups analyzed (data not shown). Consistent with the above results, the −270C-Met66 haplotype was more represented in both the AN and the BN groups when compared with controls ($P = 0.02$ and $P = 0.002$, respectively; Table 4)

ED-related phenotypes

We also examined the effect of these two sequence variants on age at onset of weight loss, minBMI and maxBMI. No

Table 1. Distribution of genotypes and alleles for the Val66Met BDNF variant in 389 patients with BN and 403 control subjects

Population	No. of genotypes (%)				No. of alleles (%)			
	Val/Val	Val/Met	Met/Met	*P* (1 d.f.)	Val	Met	*P* (1 d.f.)	OR (95 CI)
Spanish ($n = 81$)	47 (58)	31 (38.3)	3 (3.7)	0.026	125 (77.2)	37 (22.8)	0.028	1.54 (1.02–2.32)
Italian-Mi ($n = 118$)	59 (50.0)	50 (42.4)	9 (7.6)	<0.001*	168 (71.2)	68 (28.8)	<0.001*	2.10 (1.50–2.95)
Italian-Flo ($n = 97$)	61 (62.9)	31 (32.0)	5 (5.1)	0.11	153 (78.9)	41 (21.1)	0.062	1.39 (0.94–2.06)
German ($n = 93$)	58 (62.4)	33 (35.4)	2 (2.2)	0.098	149 (80.1)	37 (19.9)	0.13	1.29 (0.86–1.93)
Total ($n = 389$)[a]	225 (57.8)	145 (37.3)	19 (4.9)	<0.001*	595 (76.5)	183 (23.5)	<0.001*	1.59 (1.24–2.05)
Controls ($n = 403$)	282 (70.0)	112 (27.8)	9 (2.2)		676 (83.9)	130 (16.1)		

Mi, Milan; Flo, Florence.
*P-values statistically significant after Bonferroni correction ($P \leq 0.025$).
[a]Stratified by center.

Table 2. Distribution of genotypes and alleles for the −270C > T BDNF SNP in 389 patients with BN and 403 control subjects

Population	No. of genotypes (%)				No. of alleles (%)			
	CC	CT	TT	*P* (2 d.f.)	C	T	*P* (1 d.f.)	OR (95 CI)
Spanish ($n = 81$)	73 (90.1)	7 (8.7)	1 (1.2)	0.44	153 (94.4)	9 (5.6)	0.69	0.86 (0.41–1.82)
Italian-Mi ($n = 118$)	108 (91.5)	10 (8.5)	0	1.00	226 (95.8)	10 (4.2)	0.86	1.14 (0.56–2.33)
Italian-Flo ($n = 97$)	96 (99.0)	1 (1.0)	0	0.005*	193 (99.5)	1 (0.5)	0.003*	9.8 (1.33–71.91)
German ($n = 93$)	77 (82.8)	14 (15.0)	2 (2.2)	0.025*	168 (90.3)	18 (9.7)	0.014*	2.10 (1.17–3.77)
Total ($n = 389$)[a]	354 (91.0)	32 (8.2)	3 (0.8)	0.46	740 (95.1)	38 (4.9)	0.56	1.0 (0.62–1.56)
Controls ($n = 403$)	365 (90.6)	37 (9.2)	1 (0.2)		767 (95.2)	39 (4.8)		

Mi, Milan; Flo, Florence.
*P-values statistically significant after Bonferroni correction ($P \leq 0.025$).
[a]Stratified by center.

differences were found when patients with AN were considered (data not shown). Nevertheless, the mean maxBMI was significantly lower in the −270T carriers (23.76 kg/m^2) that in non-carriers in the BN group (25.78 kg/m^2, $P = 0.025$; data not shown). Once we reduced heterogeneity by excluding the German patients, these maxBMI differences in the BN sample were more significant ($P = 0.018$) and differences in the age of onset of weight loss were also detected ($P = 0.001$), with a younger age at onset in the −270T carriers (15.8 years) than non-carriers (18.12 years; Table 5). After Bonferroni correction only age at onset of weight loss was still positively associated to the −270C>T SNP. Conversely, when we considered the BN samples from Germany, we obtained higher mean scores of maxBMI and age at onset of weight loss in the −270T carriers than in non-carriers, but these differences did not reach statistical significance ($P < 0.1$; data not shown).

DISCUSSION

The results of this study provide evidence for association of BDNF and both AN and BN. The joint analysis of all ED populations from the different centers revealed that, over inter-population variability, there was a statistical significant excess of Met66 carriers in the AN, ANR, ANB and BN groups. When we analyzed the ED patients by centers we also found positive association between BDNF and ED in all the studied samples but, while AN was found associated to the Val66Met variant, in the group with BN we detected allelic heterogeneity, being the bulimic phenotype associated with either the Val66Met or the −270C>T SNP, depending on the population under study. As we detected no differences among controls from the different centers, this population variability may not be due to ethnic differences of the Val66Met frequencies, as previously reported (12). We also observed differences among the AN subtypes as, depending on the population analyzed, either ANR or ANB was associated to the Val66Met variant. The reduced sample size, once we subdivided patients by population and clinical subtype, may contribute to this genetic heterogeneity among the AN groups from the different centers. Since no differences among patients from different centers were found, the association between the Val66Met and ED does not seem to be influenced by the criteria used to determine the phenotypes.

Although the −270C > T SNP was associated with BN in the Florencian and German samples, the joint analysis of all centers did not reveal a positive association between this sequence variant and ED. The −270C > T promoter variant was selected for analysis on the basis of previous results of trend in association in Spanish ED patients. Spurious findings could be responsible for these positive results, but the strong association between the −270C allele and older age of onset of weight loss in BN supports the participation of this sequence variant in the bulimic phenotype, and further suggests the presence of allelic heterogeneity. Consistent with these results, a significant association between the −270C-Met66 haplotype and both AN and BN subtypes

Table 3. Distribution of genotypes and alleles for the Val66Met BDNF variant in 753 patients with AN and 510 controls

Population	AN subtype	No. of genotypes (%)				No. of alleles (%)			
		V/V	V/M	M/M	*P* (2 d.f.)	V	M	*P* (1 d.f.)	OR (95 CI)
Italian-Milan	AN ($n = 109$)	54 (49.5)	50 (45.9)	5 (4.6)	<0.001*	158 (72.5)	60 (27.5)	<0.001*	1.83 (1.3–2.57)
	ANR ($n = 42$)	19 (45.2)	20 (47.6)	3 (7.2)	0.002**	58 (69.0)	26 (31.0)	0.002**	2.16 (1.32–3.53)
	ANB ($n = 66$)	35 (53.0)	29 (44.0)	2 (3.0)	0.009**	99 (75.0)	33 (25.0)	0.02	1.61 (1.05–2.36)
Spanish	AN ($n = 87$)	48 (55.2)	37 (42.5)	2 (2.3)	0.01*	133 (76.4)	41 (23.6)	0.03	1.48 (1.01–2.19)
	ANR ($n = 40$)	19 (47.5)	20 (50)	1 (2.5)	0.006**	58 (72.5)	22 (27.5)	0.018	1.83 (1.09–3.07)
	ANB ($n = 45$)	28 (62.2)	16 (35.6)	1 (2.2)	0.23	72 (80.0)	18 (20.0)	0.3	1.2 (0.70–2.07)
Italian-Florence	AN ($n = 111$)	65 (58.6)	42 (37.8)	4 (3.6)	0.028	172 (77.5)	50 (22.5)	0.039	1.4 (0.98–2.0)
	ANR ($n = 50$)	28 (56.0)	19 (38.0)	3 (6.0)	0.05	75 (75.0)	25 (25.0)	0.038	1.61 (0.99–2.6)
	ANB ($n = 57$)	36 (63.2)	20 (35.1)	1 (1.7)	0.24	92 (80.7)	22 (19.3)	0.32	1.15 (0.70–1.89)
French	AN ($n = 163$)	95 (58.3)	60 (36.8)	8 (4.9)	0.01*	250 (76.7)	76 (23.3)	0.009*	1.46 (1.08–1.99)
	ANR ($n = 87$)	55 (63.2)	28 (32.2)	4 (4.6)	0.2	138 (79.3)	36 (20.7)	0.16	1.25 (0.83–1.86)
	ANB ($n = 76$)	40 (52.6)	32 (42.1)	4 (5.3)	0.005**	112 (73.7)	40 (26.3)	0.006**	1.72 (1.16–2.56)
British	AN ($n = 86$)	53 (61.6)	31 (36.1)	2 (2.3)	0.12	137 (79.7)	35 (20.3)	0.18	1.2 (0.8–1.8)
	ANR ($n = 35$)	23 (65.7)	10 (28.6)	2 (5.7)	0.42	56 (80.0)	14 (20.0)	0.31	1.20 (0.65–2.21)
	ANB ($n = 27$)	12 (44.4)	15 (55.6)	0	0.01**	39 (72.2)	15 (27.8)	0.04	1.85 (1.00–3.44)
German	AN ($n = 197$)	142 (72.1)	48 (24.4)	7 (3.5)	0.21	332 (84.3)	62 (15.7)	0.29	1.1 (0.8–1.52)
	ANR ($n = 93$)	68 (73.1)	21 (22.6)	4 (4.3)	0.23	157 (84.4)	29 (15.6)	0.34	1.12 (0.73–1.72)
	ANB ($n = 37$)	32 (86.5)	4 (10.8)	1 (2.7)	0.014	68 (91.9)	6 (8.1)	0.025	2.34 (1.0–5.49)
Total[a]	AN ($n = 753$)	457 (60.7)	268 (35.6)	28 (3.7)	0.0008*	1182 (78.5)	324 (21.5)	0.003*	1.37 (1.12–1.65)
	ANR ($n = 347$)	212 (61.1)	118 (34.0)	17 (4.9)	0.003**	542 (78.1)	152 (21.9)	0.004**	1.43 (1.15–1.77)
	ANB ($n = 308$)	183 (59.4)	116 (37.7)	9 (2.9)	0.012**	482 (78.2)	134 (21.8)	0.035	1.29 (1.03–1.62)
Controls	($n = 510$)	350 (68.6)	145 (28.4)	15 (3.0)		845 (82.8)	175 (17.2)		

Subtypes of ANR and ANBP were also considered.
*P-values statistically significant after Bonferroni correction ($P \leq 0.025$).
**P-values statistically significant after Bonferroni correction ($P \leq 0.0125$).
[a]Stratified by center.

Table 4. Haplotype distribution of the −270C > T and the Val66Met polymorphisms in ED patients and controls

Haplotype	−270C > T	Val66Met	No. of cases (%)	No. of controls (%)
BN ($n = 389$)[a]				
1	−270C	Val66	558 (71.7)	637 (79)
2	−270T	Val66	37 (4.7)	39 (4.9)
3	−270C	Met66	182 (23.3)	130 (16.1)
4	−270T	Met66	1 (0.13)	—
AN ($n = 753$)[b]				
1	−270C	Val66	1114 (74)	793 (77.8)
2	−270T	Val66	68 (4.5)	52 (5.1)
3	−270C	Met66	324 (21.5)	175 (17.1)

[a]$\chi^2 = 14.45$; $P = 0.0024$.
[b]$\chi^2 = 7.4$; $P = 0.02$.

Table 5. Mean and standard deviations of age at onset of weight loss (AO), minBMI and maxBMI according to the Val66Met and −270C > T variants in patients with BN

Phenotypes	Genotype	*N*	Mean	SD	*P*-value[a]	95% CI
AO (years)	V/V	197	18.1	4.6	0.82	−0.94 to 1.17
	V/M or M/M	120	17.9	4.2		
	C/C	253	18.1	4.5	0.001*	1.03 to 3.50
	C/T or T/T	15	15.9	2.0		
minBMI (kg/m^2)	V/V	158	20.2	3.8	0.56	−0.63 to 1.16
	V/M or M/M	120	19.9	3.7		
	C/C	261	20.0	3.8	0.52	−0.73 to 1.40
	C/T or T/T	18	19.7	2.0		
maxBMI (kg/m^2)	V/V	157	25.4	4.5	0.07	−2.24 to 0.09
	V/M or M/M	122	26.4	5.2		
	C/C	262	26.0	4.9	0.018*	0.44 to 4.25
	C/T or T/T	18	23.6	3.7		

*P-values considered statistically significant ($P < 0.05$).
[a]*t*-Test for two independent samples (Val66 carriers versus non-carriers and −270T carriers versus non-carriers).

was detected. A large-scale analysis with a set of over 50 SNPs will be performed in several European populations and should provide further insight into the relationship between this gene and ED.

We also observed that the German population of ED displayed a different genetic background in comparison with the other groups, showing an overrepresentation of the −270T and the Val66 alleles instead of the −270C and the Met66 variants, both in excess in the ED patients from the other populations. The possibility of diagnostic differences or a population-origin phenomenon is unlikely to be responsible for these differences as these European samples have been analyzed for other markers and no differences among centers have been identified (11,13,14). The above results could be explained by other mechanisms. First, the −270C and Met66 alleles may directly, either independently or together, confer susceptibility to ED. In the German samples, where association was found with the opposite *BDNF* alleles, an as yet unknown *BDNF* susceptibility variant in linkage disequilibrium with the −270T-Val66

haplotype could participate directly in the predisposition to AN and BN. Alternatively, as linkage disequilibrium patterns differ among populations, the −270C>T and Val66Met sequence variants could not have functional consequences, but a new *BDNF* variant in linkage disequilibrium with these two SNPs could be the responsible for the ED susceptibility. Thus, while in the German population this susceptibility allele would be in linkage disequilibrium with the −270T-Val66 haplotype, in the other populations it would be associated with the −270C-Met66. Nevertheless, as the *BDNF* gene was screened for mutations or SNPs in 95 Spanish patients and only two single mutations were identified in two AN samples (11), linkage disequilibrium to a third nearby *BDNF* susceptibility allele is unlikely to be the involved in the susceptibility to AN and BN. Interestingly, another candidate gene, *MALS3*, is located 140 kb upstream of *BDNF*. *MALS3* is a member of a family of mammalian homologs of the *Caenorhabditis elegans* LIN-7 (15) and encodes for a protein with an essential role in recruiting receptors and enzymes to specific synaptic sites (16). It is possible that some *MALS3* SNPs could be in linkage disequilibrium with *BDNF* SNPs, determining a risk haplotype participating in the etiology of ED in a similar way as in other complex disorders such as the cytokine gene cluster and susceptibility to Crohn's disease (17). Thus, the mutation screening of the *MALS3* gene and the analysis of a large collection of markers in the region would be necessary to determine its possible involvement in AN or BN.

We also detected the effect of the −270C variant on old age of onset of weight loss and high maxBMI in the BN sample. Nevertheless, *BDNF* did not remain positive associated to this BMI extreme condition after considering the multiple comparison correction. However, the Bonferroni correction, taking into account two polymorphisms, two AN subtypes and three ED-related phenotypes, may be too stringent to detect the effect of a susceptibility gene in a complex phenotype such as ED.

No evidence for functional consequences of the −270C>T polymorphism has been described, but it was reported recently that the Val66Met SNP determines functional changes in hippocampal neurons by attenuating the regulated pathway that secretes BDNF in response to neuronal activity (18). Moreover, this sequence variant seems to be a good candidate to participate in the etiology of ED through its effect on the function and the stability of the BDNF precursor, which is extracellular secreted and biologically active (20), or on its processing to the mature neurotrophin. Considerable evidence indicates that alterations in the BDNF system may predispose to AN or BN. Thus, BDNF serum levels are decreased in ED patients when compared with controls (21), and intraventricular administration of BDNF in rats induced a severe dose-dependent appetite suppression, weight reduction and increased hypothalamic 5-hydroxyindoleacetic acid levels (3). Interestingly, this hyperserotoninergic function has been detected in long-term weight restored BN patients and is considered a trait marker in ED (22,23). Likewise, murine models also corroborate the relationship between alterations in BDNF and abnormalities in eating behavior. Thus, BDNF knock-out mice develop a tendency toward obesity with serotonergic dysfunctions, hyperphagia and significant weight gain (5–7). Moreover, the association between BDNF and ED is also supported by the role of this neurotrophin as a key mediator in the neuronal responses to external environmental factors such as dietary restriction or exercise (19,24–28), and by its contribution to other psychiatric disorders such as bipolar disorder, obsessive–compulsive disorder and personality facets associated with depression (29–32).

In conclusion, the results of our study show that, over inter-population variability, BDNF participates in the predisposition to both AN and BN and age of onset of weight loss. Although an effect of BDNF on high maxBMI was lost once the data was adjusted for multiple testing, this association also remains possible. This is the first study that identifies a susceptibility gene involved in the etiology of ED across different populations, and demonstrates the importance of using large samples in association studies to detect a genetic effect in a complex phenotype. At this stage it remains uncertain whether both the Val66Met and −270C>T SNPs are independent risk factors or they interact to confer susceptibility to AN and BN. In addition, it could also be possible that other BDNF SNPs are associated with ED, since several *BDNF* nucleotide changes, one of them affecting splicing, were detected in Spanish samples (11). A complete characterization of SNPs within and flanking the *BDNF* locus and further studies of other sets of patients and ED trios should provide additional clues about the genetic weight of this neurotrophic factor in the predisposition to ED.

MATERIALS AND METHODS

Study subjects

The clinical sample consisted of 1142 Caucasian patients with ED consecutively recruited in six European centers participating in the EC Framework V 'Factors in Healthy Eating' consortium QLK1–1999-916 (France, Germany, Italy, Spain and UK) between November 1997 and August 2002. All subjects fulfilled DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th edn) criteria for ED and were diagnosed using various types of interview according to the country. The Spanish and Florence samples were diagnosed with the Structured Clinical Interview for Mental Disorders, research version 2.0 (SCID-I), the British sample with the ATE EAT, the French patients with the Diagnostic Interview for Genetic Studies (DIGS), the Italian sample from Milan with the Diagnostic Interview Schedule-Revised (DIS-R) and the German sample with the Composite International Diagnostic Interview (CIDI). The group consisted of 389 BN cases (34%; 251 binge-eating/purging BN, seven non-purging subtype BN and 131 BN cases not classified as binge-eating/purging or non-purging subtype) and 753 AN cases [66%, 347 restricting subtype, 308 AN/BP and 98 AN cases with insufficient time elapsed (<3 years restricting illness) to be classified as restricting or binge-eating/purging subtype]. Diagnosis was blind to genotype. Although some past clinical traits were difficult to reliably recall, clinical information was available from most of the patients. Thus, 865 patients were assessed for age at onset of weight loss, 924 for minBMI and 702 for maxBMI. Most of patients were female ($N = 96\%$) and have been studied in previous reports (11,13,14). The average age at assessment was 23.6 years

Table 6. Distribution of subjects according to population, gender and diagnosis of seven European samples of patients with ED studied for the BDNF Val66Met and −270C/T SNPs

Population	AN			BN			Controls		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
Spanish	8	79	87	5	76	81	12	211	223
Italian-Florence	5	106	111	2	95	97	1	67	68
Italian-Milan	5	104	109	3	115	118	0	58	58
British	1	85	86	—	—	—	5	46	51
German	10	187	197	3	90	93	5	49	54
French	3	160	163	—	—	—	0	56	56
Total	32	721	753	13	376	389	23	487	510

(SD = 8.9) for AN patients and 25.3 years (SD = 7.3) for BN patients. The lifetime minBMI was 14.21 kg/m^2 (SD = 2.28) for AN patients and 19.8 kg/m^2 (SD = 3.9) for BN patients. The lifetime maxBMI was 20.47 kg/m^2 (SD = 3.75) for AN patients and 25.6 kg/m^2 (SD = 5.2) for BN patients. The control sample consisted of 510 Caucasian unrelated subjects matched for ethnicity and sex [95.5% females ($N = 487$)] recruited from the six European centers. The distribution of the 1652 subjects according to gender, diagnosis and population group is summarized in Table 6.

Molecular analysis

Two SNPs in the *BDNF* gene were typed from each DNA sample by the polymerase chain reaction (PCR) in a total volume of 10 μl containing 50 ng of template DNA, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 200 μM of dNTP (Pharmacia Biotech), 10 pmol of each oligonucleotide (Life Technologies) and 0.25 U of *Taq* DNA polymerase (Boehringer Mannheim). Amplification conditions consisted on an initial 4 min denaturation step at 94°C, 32 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, followed by a final extension of 10 min at 74°C. The −270C>T sequence variant is located in the 5′ untranslated region of the gene and changes a *Hin*fI restriction site. Amplification was performed with primers 5′-GAGCCAGAATCGGAACCACG-3′ and 5′-TCTACCGGAGGGGAGGAAAG-3′. Depending on the absence or presence of the polymorphic *Hin*fI restriction site, either two fragments of 190 and 7 bp (−270C) or three fragments of 157, 33 and 7 bp (−270T) are detected. The Val66Met SNP changes a *Nla* III restriction site. Primer sequences were 5′-AGGTGAGAAGAGTGATGACC-3′ and 5′-CTGGACGTGTACAAGTCTGC-3′. In the presence of the Met66 allele, digestion with *Nla* III produces four fragments of 160, 59, 58 and 15 bp, whereas the Val66 allele produces five fragments of 83, 77, 59, 58 and 15 bp. Both SNPs were resolved on 12% polyacrilamide gels (19 : 1).

Statistical analysis

Average minBMI, maxBMI, age of assessment and age of onset of weight loss in each studied group were measured by the statistical package SPSS 10.0. The distribution of genotypes for the different populations was tested for the Hardy–Weinberg equilibrium by a χ^2 analysis using the INSTAT Graphpad software. Patients with ED were grouped according to the clinical subtype in AN or BN for all statistical tests. Under the hypothesis that BDNF may confer susceptibility to different AN subtypes in different ways and to reduce heterogeneity, AN patients were also subdivided as restricting (ANR) or binge-eating/purging anorexia (ANBP) for the statistical analysis.

Case–control study

The power analysis was performed *post hoc* on the ED groups from each population with the Power Calculator software (Department of Statistics of the University of Los Angeles; http://calculators.stat.ucla.edu/powercalc), assuming a lifetime risk of 2% and a significance level of 0.05. The case–control study was carried out comparing both genotype and allele frequencies between patients from each population and the total sample of controls ($N = 510$ for AN, and $N = 403$ for BN). To avoid the confounding effect of population, the combined case–control analysis of all samples was stratified by center using the Epi Info 2000 package (http://www.cdc.gov/epiinfo/index.htm). As the aim of this study was to replicate a previously reported association between the Met66 allele and ED (11), the direction in which the association may occur was previously established. For this reason, the comparison of both allelic and genotypic frequencies of the Val66Met SNP was assessed by one-tailed Fisher's exact test under a dominant model. Since no direction in the association for the −270C>T variant was expected a priori, the comparison of genotypes and allele frequencies for this SNP was performed by two-tailed Fisher's exact test. Both statistical tests were performed using the statistical package SPSS 10.0. Since two genetic markers were analyzed, we used the Bonferroni correction for χ^2 tests and corrected P-values were considered to be statistically significant for $P < 0.025$. Nevertheless, given that AN patients were also subdivided ANR and ANBP, P-values were also corrected by Bonferroni in these groups and significance was set at $P \leq 0.0125$. The effect of population on the Val66Met and −270C>T carrier status was evaluated in both patients and control groups using the stepwise logistic regression analysis of the statistical package SPSS 10.0. Linkage disequilibrium tests were performed in both case and control groups using the PMPLUS program (33). The total number of cases exceeded the maximum number of individuals that the PMPLUS

software is capable of analyzing. Patients were randomly subgrouped in two files and the overall linkage disequilibrium score resulting from the mean of the ones was obtained in each single group. Haplotype estimations from the population genotype data were performed using the PHASE version 2.0 software (34,35). Mean scores of minBMI, maxBMI and age of onset of weight loss were compared between patients carrying the Met66 allele and those homozygous for the wild-type allele, and −270T carriers versus −270T non-carriers, by *t*-tests using the statistical package SPSS 10.0. After Bonferroni correction, considering two SNPs and three ED-related phenotypes, levels below 0.008 were considered significant.

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