comparison of BDNF levels in 50 ED discordant sib-pairs showed an increased risk for ED in parallel with BDNF blood levels (OR high levels = 2·29; OR very high levels = 9·65, p trend = 0·007).
Interpretation Our data demonstrate that altered BDNF levels caused by BDNF gene variability confer susceptibility to ED. These findings provide physiological evidence for a role of BDNF in the development of AN and BN, and strongly argue for its involvement in eating behaviour and body weight regulation.

Altered brain-derived neurotrophic factor blood levels and gene variability are associated to anorexia and bulimia

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Summary

Background Murine models and association studies in eating disorders (ED) patients have suggested a potential role of the brain-derived neurotrophic factor (BDNF) in eating behaviour as an anorexigenic factor. Intraventricular infusion of BDNF in rat causes hypophagia, while disruption of the BDNF gene leads to hyperphagia and obesity. In humans, the -270C/T BDNF Single Nucleotide Polymorphism (SNP) is associated to bulimia (BN), and the Val66Met variant is associated to both BN and anorexia (AN) in different populations.

Methods To further test the anorexigenic role of this neurotrophin in humans we have screened 36 SNPs in the BDNF gene and tested for their association with ED and plasma BDNF levels as a quantitative trait. We have performed a family-based association study in 115 ED nuclear families and analyzed BDNF blood levels in 110 ED patients and in 50 sib-pairs discordant for ED.

Findings The rs7124442T/rs11030102C/rs11030119G haplotype was found associated to high BDNF levels (mean BDNF TCG haplotype carriers = 43·6 ng/ml vs. mean others 23·0 ng/ml p = 0·036) and BN (Z = 2·64; pdom = 0·008), and the rs7934165A/270T haplotype was associated to AN (Z = -2·64; p = 0·008). The comparison of BDNF levels in 50 ED discordant sib-pairs showed an increased risk for ED in parallel with BDNF blood levels (OR high levels = 2·29; OR very high levels = 9·65, p trend = 0·007).

Interpretation Our data demonstrate that altered BDNF levels caused by BDNF gene variability confer susceptibility to ED. These findings provide physiological evidence for a role of BDNF in the development of AN and BN, and strongly argue for its involvement in eating behaviour and body weight regulation.

Introduction

Anorexia nervosa (AN) and bulimia nervosa (BN) are complex psychiatric conditions in which genetic and environmental factors are involved.[1,2] Among those genes with a role in satiety, appetite and weight regulation, brain-derived neurotrophic factor (BDNF) is likely to participate in the pathophysiology of eating disorders (ED). BDNF encodes a neurotrophic factor with an essential role in neuronal development and synaptic plasticity.[3,4] Physiological and animal models suggest that increased levels of this neurotrophic factor in the central nervous system (CNS) could participate in the aetiology of ED. The administration of BDNF in the CNS induces weight loss and appetite suppression, while BDNF knock-out mice develop obesity and hyperphagia.[5-8] The same murine obese phenotype has been observed when the expression of the BDNF high affinity receptor, NTRK2, is reduced in the hypothalamus.[9] In addition, a patient with severe obesity and developmental delay with a de novo NTRK2 mutation has been reported.[10] Finally, in mice, 48 hours of fasting reduces BDNF levels in the arcuate nucleus, while chronic dietary restriction increases BDNF levels in the brain.[9,11,12]

BDNF levels in plasma or serum have been analyzed for several psychiatric disorders, such as schizophrenia, panic disorder, depression, and ED, finding altered blood levels in most of these disorders, including in ED patients.[13-18]

Genetic studies have reported significant and consistent association of BDNF variants with ED.[19,20] The -270C/T BDNF single nucleotide polymorphism (SNP) is associated to BN, and the Val66Met variant to both AN and BN in different European populations.[21,22] In addition, we have detected a significant

association of NTRK2 to AN, minimum body mass index (BMI), and harm avoidance.[23] With respect to BDNF, the direct relationship of the Val66Met variant in the development of ED and its potential functional consequences are still unclear[24].

In order to determine if other variants in tight linkage disequilibrium with this SNP configure an extended functional haplotype involved in the susceptibility to ED, we performed a family-based association study (FBAT) for 36 SNPs covering the BDNF gene in a sample of 115 ED nuclear families. Since these SNPs in the BDNF gene are likely to influence BDNF protein levels in brain and a positive correlation between serum and cortical BDNF levels has been described in rats,[25] we also hypothesized that some genetic variants in the BDNF gene may be accompanied by altered blood BDNF levels. We first tested if there were variants associated to plasma BDNF levels in a sample consisting of 110 ED patients and further compared plasma BDNF levels in 50 ED patients with respect to their respective 50 available siblings. Our data demonstrate that altered BDNF levels, caused by BDNF gene variability, confer susceptibility to ED, providing physiological evidence for a role of BDNF in the development of AN and BN.

Methods

Subjects

All patients with ED used for the study were Spanish and with a Caucasian origin and were consecutively admitted in the Psychiatric Unit of the Hospital de Bellvitge, between 1999 and 2002. The clinical sample used for the family-based association study consisted of 115 nuclear families (51 trios and 64 families with at least one unaffected sib-pair) with ED patients (49 AN, 57 BN, 9 ED not otherwise specified or EDNOS). The clinical subgroup used to analyse BDNF plasma levels consisted of 110 patients, 49 AN cases (44·5%; 25 restricting AN (ANR), and 24 binge-eating/purging subtype (ANP)) and 61 BN patients (55·5%; 58 binge-eating purging (BNP) and three non-purging subtype (BNNP)). All patients were female, fulfilled DSM-IV criteria for ED and were diagnosed using the Structured Clinical Interview for Mental Disorders, research version 2·0 (SCID-I). More than three years of restrictive illness were necessary to classify patients as ANR. The lifetime minimum BMI was 15·3 kg/m^2 (SD = 1·37) for AN patients and 19·2 kg/m^2 (SD = 2·8) for BN patients. The average age at assessment was 24·5 years old (SD = 6·0) for AN and 25·8 years old (SD = 5·0) for BN. The average age of onset of weight loss was 15·3 years old (SD = 4·3) for AN, and 19·1 years old (SD = 4·6) for BN. Diagnosis was blind to genotype and most of the patients have been described in previous reports.[19,21-23,26] The control group for the sib-pair analysis consisted of healthy sisters of 50 ED patients. The study had ethics approval from the Bellvitge Hospital research ethics committee. We obtained written informed consent from all participants.

Quantification of plasma BDNF levels

Plasma was obtained by centrifugation from fresh peripheral blood samples and

stored at -20ºC until used for the assay. For the sib-pairs, samples were extracted at the same time point and patients were not following any treatment at the time of blood extraction. BDNF levels were measured by the enzyme-linked immunoassay system (ELISA; BDNF Emax Immunoassay System kit (Promega, Madison, WI), following manufacturer's instructions.

BDNF levels in unaffected sib-pair analysis were compared by paired t-test. Then, BDNF levels were ranked in the following tertiles: basal (lower than 31·12 ng/ml), high (between 31·12 and 58·32 ng/ml) and very high (higher than 58·32 ng/ml). Cox-time dependent logistic regression was performed taking the first tertil as a reference to estimate odds ratios.

Selection of BDNF SNPs

All the SNPs that were available at the time of the study design were obtained from Celera SNP database (http://www.celera.com) and from dbSNP build134 database (http://www.ncbi.nlm.nih.gov/SNP) and covered a region of 63·8 kb encompassing all the BDNF gene. Only SNPs with a unique mapping location on the NCBI build34 genome assembly were considered for further analysis. The SNPs were submitted to the Applied Biosystems design pipeline to select those ones that passed the design and genomic rules, ending up with the synthesis of ligation probes for 36 SNPs. Five SNPs were located in the coding exon, and two of them were located in 5' untranslated exons. The other SNPs were located in intron 2 of BDNF. Mean distance between markers was 1·8 kb.

Molecular analysis

All SNPs were genotyped using the SNPlex™ platform (Applied Biosystems), which is based on multiplex OLA/PCR and capillary electrophoresis, as described by De la Vega et al.[27] For the genotyping process we followed the

manufacturer's protocol for the SNPlex™ Multiplex Genotyping Systems (Applied Biosystems, Foster City, CA). Briefly, 12 μl of DNA at 100 ng/μl was fragmented by boiling at 99ºC for 10 minutes, two μl were dispensed in a 384 microplate and the manufacturer's specifications were followed through phosphorylation, oligonucleotide ligation, exonuclease clean-up, PCR, and hybridization steps using various robotic systems.

Family-based association study

Hardy-Weinberg equilibrium, and linkage disequilibrium of SNPs was assessed by the *Haploview* v2·03 software (http://www.broad.mit.edu/mpg/haploview/).[28] To avoid multiple testing and redundant genetic information, we first selected tagSNPs using the tagger algorithm implemented in the *Haploview* software that generates marker sets that are captured by *tagSNPs*. Nine tagSNPs for different marker sets (Supplementary Table S1), capturing all the allelic variation within the gene, were selected with a minimum R^2 of 0·9[28] (The family-based association study was performed using *FBAT* software as described in Laird, et al.[29] The genotype model was used to first screen for association with the tagSNPs. When a tagSNP was found to be associated to the disease, the rest of the SNPs in the marker set were analysed. Haplotype analysis was performed with *hbat* algorithm of the *FBAT* software, which computes haplotypes before applying executing *FBAT* using bi-allelic and multi-allelic modes. The best fitting model among additive, dominant or recessive was chosen based on the lowest p value obtained.[30]

Effect of the SNPs in plasma BDNF levels

BDNF plasma levels were logarithm transformed in order to have normality and homogeneity of variance among all the groups. Box plots representing the

BDNF levels of different genotypes of every SNP were represented to check possible association between SNPs and plasma BDNF levels. This relationship was then analysed using a general linear model. For each SNP genotype we computed the mean and its 95% confidence interval. In all cases, the homozygosity for the major allele among controls was set as the reference class. Analyses were done under a codominant model (three genotypes separated), a dominant model (heterozygotes grouped with the homozygotes for the rarer allele), a recessive model (heterozygotes grouped with the homozygotes for the common allele), an overdominant model (homozygotes both grouped), and an additive model (genotypes coded as numeric values being "1", homozygous for the common allele, "2", heterozygous, and "3", homozygous for the rare allele). P values were derived from likelihood ratio tests. The best model was chosen using the Akaike Information Criteria. Bonferroni method was used to correcte for multiple comparisons. All analyses were carried out using *SNPassoc* R library.[31] Haplotypes were reconstructed using the *PHASE* software version 2·0,[32,33] and a global test of hypothesis for every haplotype was done using a lineal model.

Pre-albumin concentration assessment

Serum pre-albumin concentrations were analyzed in 105 ED patients and 46 unaffected sisters by immunoturbidimetry (ITC Diagnostics, Barcelona, Spain) in an ILab 1600 automated chemistry analyzer (Instrumentation Laboratories, Milan, Italy). The levels between patients and controls were compared by paired t-test. Pearson Correlation was used to test the link between prealbumin and BDNF levels.

Results

Haplotype structure of the BDNF gene

As tagSNPs may be population specific, it has been suggested that they should be newly assessed in the local population where the association study is going to be performed.[34-36] Thus, a total of 36 SNPs covering the BDNF region were selected and genotyped in 115 nuclear families of ED patients (49 AN, 57BN, 9 EDNOS). Thirteen of the SNPs were discarded because they were either monomorphic or had minor allele frequencies lower than 1%. The remaining variants were further checked for Hardy Weinberg equilibrium, and 22 SNPs could be finally included in the analysis to determine the linkage disequilibrium (LD) structure of the region. Using the four-gamete rule, the region encompassing BDNF is composed of three LD blocks. The larger block spans 44 kb and encompasses 15 SNPs, including the coding region of BDNF and the functional Val66Met variant (rs6265). The other two blocks are smaller and include the 3'UTR and the BDNF 5' flanking region, respectively (Supplementary Figure S1). From the 22 polymorphisms in the region we selected 9 tagSNPs, which captured the allelic variation within the gene with a minimum R^2 of 0·9 (Supplementary Table S1).

Family-based association study

We performed a family-based association study using the *FBAT* software considering the genotypes of nine tagSNPs in a sample of 115 nuclear families (51 trios and 64 families with at least one unaffected sib). As no significant association was found for any of the tag SNPs when ED was considered using the genotype model, comparing the expected vs. observed transmission of each possible genotype, we further stratified the sample according to the ED subtype.

Anorexia Nervosa

In the anorexia subgroup (49 nuclear families), an undertransmission of the AA genotype (Z = -2·52; p = 0·01) of the rs7934165 SNP, tag for marker set 2 and an overtransmission of the CT genotype (Z = 2·04; p = 0·04) of the -270 C/T SNP, tag for marker set 4 were found associated to the disease, although statistical significance was lost after Bonferroni correction (Supplementary Table S2). In a subsequent step, we performed haplotype analyses. The two-locus haplotype containing both tagSNPs revealed undertransmission of haplotype AC under an additive model (Z = -2·64; p = 0·008) (Table 1). This AC haplotype includes four SNPs contained in marker set 2, and three SNPs included in marker set 4 (Supplementary Table S1).

Bulimia Nervosa

In the case of BN patients (57 nuclear families) an overtransmission of genotypes CC (Z = 2·33; p = 0·02) of rs11030102, tag for marker set 3, GG (Z = 2·29; p = 0·02) of rs11030119, tag for marker set 5 and TT (Z = 3·07; p = 0·002) of rs7124442, tag for marker set 6, was observed. Only the overtransmission of the TT genotype for rs7124442 remained statistically significant after Bonferroni correction considering 16 tests (Suplementary Table S3). Consistently, the most common haplotype for these three tagSNPs showed an overtransmission of the CGT haplotype assuming a recessive model (Z = 2·64; p dominant = 0·008) (Table 2). The haplotype includes three SNPs of marker set 3, two of marker set 5, and two of marker set 6 (Supplementary Table S1).

Relationship between SNPs and BDNF plasma levels

The genotype distribution of all BDNF SNPs was compared to plasma protein levels determined in a total of 110 ED patients. SNPs, rs7124442 (p additive =

0·0006), rs1030102 (p recessive = 0·001), rs2049045 (p additive = 0·04), rs11030107 (p recessive = 0·002), rs10835210 (p recessive = 0·01), rs7103873 (p dominant = 0·004), rs7127507 (p recessive = 4·7 E^{-5}), -270 C/T (p additive = 0·004), rs2030324 (p dominant = 0.002), rs1030119 (p recessive = 4·7 E^{-5}), rs7934165 (p dominant = 0·004), rs10767665 (p dominant = 0·003), and hCV7469136 (p recessive = 0·0001), where associated to BDNF plasma levels (Figure 1 and Supplementary Table S4). The same trend was observed when stratifying for AN or BN status (Supplementary Figures S2 and S3). When considering the unaffected sibs alone, association was found for rs712442 (p additive = 0·04), rs10835210 (p dominant = 0·002), rs7103873 (p dominant = 0·004), rs2030324 (p dominant = 0·004), rs7934165 (p dominant = 0·004), and rs10767665 (p dominant = 0·002) (Supplementary Table S5 and Supplementary Figure S4).

Relationship between ED associated haplotypes and BDNF plasma levels

We compared the effects of the BDNF haplotypes associated to AN and BN on BDNF plasma levels. No differences in BDNF levels were detected when the AN patients carrying the haplotype associated to AN were compared to the other AN patients. However, the BN patients carrying the overtransmitted haplotype in BN had significantly higher BDNF levels than non-carriers, explaining 10·7% of the variability (mean TCG carriers = 43·6 ng/ml vs. mean others 23·0 ng/ml p = 0·016) (Table 3).

Analysis of BDNF plasma levels in eating disorders discordant sib-pairs

We assessed BDNF protein levels in 50 ED patients and in their respective available unaffected sib-pairs as a control group. We found significantly higher levels in ED patients relative to their sibs (mean 52·7 ng/ml vs. mean 41·0

ng/ml, respectively; p = 0·004). In a subsequent step, we divided the sib-pairs group according to the clinical subtype of the affected cases, and the differences remained significant only in the AN sample (mean 57·8 ng/ml vs 42·6 ng/ml; p = 0·02) (Supplementary Table S6). According to the protein levels and ranking in three tertiles from basal (lower than 31·12 ng/ml), high (between 31·12 and 58·32 ng/ml), or very high levels (higher than 58·32 ng/ml), the risk of ED (AN and BN) increased in parallel with the BDNF levels. Thus, compared with subjects in the lowest tertile, subjects in the medium tertile had more than a two-fold risk for ED (OR = 2·29), while subjects in the highest tertile had more than a 9-fold risk (OR = 9·65), with an overall p trend of 0·007. This effect was still observed once we separately considered the AN and BN subgroups, although the risk was higher in the case of AN patients (Figure 2).

Correlation of BDNF levels with Body Mass Index and pre-albumin levels

Correlation between BDNF levels, and pre-albumin levels, a nutritional status marker, was assessed to discard unpaired nutritional status as a cause of the higher BDNF levels found in ED patients. Mean prealbumin levels were lower in ED patients than in its unnaffected sisters (mean ED = 0·20 g/L, mean unaffected sibs = 0·26, p = 5·3 E^{-5}), although they were not out of the reference values (0·10 - 0·4 g/L). However, there was no correlation between BDNF levels and pre-albumin (Pearson correlation = 0·016, p = 0·86). No correlation was found between BDNF blood levels and BMI (data not shown).

Discussion

Our study provides new insights into the influences of genetic variation of the BDNF gene on the susceptibility to AN and BN, and on the regulation of BDNF blood levels. Our family-based association study considering tagSNPs did not show a common haplotype associated to ED as a single group. However, a stratified analysis for each ED subtypes revealed significant associations for both AN and BN, but the haplotype composition was different for each phenotype. This result raises the possibility that different susceptibility variant(s) could be involved in the development of each phenotype. This is not surprising if we take into account the fact that, although they are considered ED subtypes, AN and BN show different physiological and psychopathological trait characteristics.[37,38] Although the variability in the BDNF gene seems to be altering eating behaviour, the biological consequences might be slightly different, evolving to either AN or BN, depending on the specific genetic variant.

The fact that other SNPs in the BDNF gene show stronger associations with ED than Val66Met and -270C/T, supports that additional causative variants, probably in LD with them, which might contribute to the phenotypes. However, since many of the associated SNPs pertain to the same LD block, it is difficult to define a critical susceptible variant conferring risk to either AN or BN. To refine the region and find stronger associated variants, a larger sample would be needed, together with a re-sequencing effort, which should also allow us to capture association with rare variants.

We have also investigated the BDNF blood levels in 110 ED patients and 50 discordant sib-pairs. Several SNPs are associated to BDNF blood levels, in ED patients, irrespective to the AN or BN status. Most of these SNPs are also

associated to BDNF blood levels when considering only the group of unaffected sibs. The low power, due to the small number of controls (n = 50), might explain the lack of statistical significance for some SNPs that are associated to BDNF blood levels in ED patients but not in controls (Supplementary Table S5 and Supplementary Figure S4). Apart from this significant global association using single markers, we also investigated the BDNF levels of carriers of the haplotypes associated to AN or BN. There were no differences in BDNF levels in the AN patients depending on the AN associated haplotype. However, in the case of BN, the same haplotype that confers susceptibility risk to the phenotype is also associated to higher BDNF levels. This last haplotype explains 11% of the variability of BDNF levels, and a mean difference between carriers and non-carriers of 20·6 ng/ml. This finding suggests that, in the case of BN, genetic variation in the BDNF gene confers risk to the development of BN through increased blood BDNF levels. In the case of AN, the haplotype might predispose to the disease in a different way, rather than altering BDNF blood levels.

Finally, we found that BDNF plasma levels are about 25% higher in ED patients than in their unaffected sibs, with an odds ratio for ED that increases in parallel with BDNF plasma levels. These results are consistent with previous findings showing that CNS intraventricular administration of BDNF induces starvation and body weight loss, while BDNF heterozygous mice develop obesity and hyperphagia.[6-8] Accordingly, increased BDNF plasma levels could reflect high protein levels in the CNS that would alter eating behaviour in ED patients. Alternatively, taking into account that BDNF expression is induced by long food restriction periods, the altered plasma levels could also reflect the

eating patterns of ED patients, more than a direct cause of the development of the disease. However, we did not find a correlation between BDNF and BMI nor pre-albumin levels, a marker of nutritional status. This argues against impaired nutritional status or altered eating patterns as the main course of the observed BDNF differences. The analysis of BDNF concentration after weight recovery, in the case of AN, should provide additional clues about the BDNF contribution to the pathogenesis of ED.

Despite the robustness of our findings regarding plasma BDNF protein levels, which support murine and association data,[5-7,19,21,22] the results challenge previous reports, where BDNF serum concentration was found decreased in AN patients.[13-15] However, these research groups measured serum BDNF levels, while we determined plasma BDNF levels. This fact may not be relevant, nevertheless, there is a source of discrepancy, since it has recently been reported that there is a poor correlation between BDNF levels in plasma and in platelets, which are the main source of BDNF in serum, and represent a long-term marker of varying plasma BDNF levels.[39] Another reason for this discrepancy could be the relatively small sample size in these studies, or the fact that serum BDNF levels in some of their control groups[13] were out of the expected range according to those of other reports.[20,40,41] In addition, it is well known that short term fasting reduces BDNF levels in the hypothalamus, while long dietary restriction periods increases the expression of this neurotrophin in the CNS.[9,11] These results suggest that the observations reported by Monteleone et al. (2004), where blood collection was performed after overnight fasting, could reflect the negative effects of short fasting periods on BDNF levels and have masked the basal BDNF levels in both ED patients

and controls. In our study, blood samples were withdrawn during the morning without neither fasting of patients nor controls, to avoid the short-term fasting response of BDNF. Else than a substantially larger sample in comparison with other reports, the control sample we used here consisted of unaffected sib pairs, which avoids the potential stratification effects and allows the comparison of each ED patient with its own sib. Indeed, blood samples from each sib-pair were collected the same day and all specimens were equally treated before the analytical procedures, which reduces possible environmental and assessment effects on the observed results.

In summary, our findings confirm the role of BDNF gene variability in ED, through, at least in part, the modulation of plasma BDNF levels. It will be important to elucidate which variants are causative, and to disclose if BDNF is a biological marker of the disease. All this together, the analysis of genetic variants and blood levels in ED provides a better understanding of the mechanisms by which BDNF regulates energy balance and feeding behaviour.

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Author's contribution:

All authors declare that have participated in the following parts of the study:

Xavier Estivill: Study design, evaluation of the data and writing the manuscript.

Josep M Mercader: BDNF analysis, data analysis and writing the manuscript.

Marta Ribasés: genotyping data, data analysis and writing the manuscript.

Mònica Gratacòs: Study design, data analysis and writing the manuscript.

Juan R González: Statistical design, data analysis and writing the manuscript.

Mònica Bayés: Genotyping analysis and data analysis.

Rafael de Cid: Genotyping analysis and data analysis.

Anna Badía: Clinical evaluation, data analysis.

Fernando Fernández-Aranda: study design, data analysis and writing the manuscript.

All authors have seen and approved the final version of the manuscript.

The corresponding author had **full access to all the data** in the study and had final responsibility for the decision to submit for publication.

None of the authors have any **conflict of interest** regarding the data submitted in this paper.

The **funding source** was used to *the* study design, collection, analysis, interpretation of data, and writing the report.

There was **no medical writer or editor** involved in the creation of your manuscript

All patient samples were collected with the **approval of the ethics committee** and written informed consent was obtained from all subjects participating in the study.

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Figure legends

Figure 1. Box plot representing BDNF plasma levels for BDNF SNPs analysed under codominant, dominant, and recessive models eating disorders patients. SNPs are ordered by genomic location in the BDNF gene. SNPs shown in blue are those that show mean statistically different BDNF plasma levels depending on the genotype. *Statistically significant after Bonferroni correction ($p<0{\cdot}002$). P values are based on logarithm of the concentration and are adjusted by anorexia or bulimia status. A, common allele; a, rare allele.

Figure 2. Odds ratio corresponding to high levels (between 31·12 and 58·32 ng/ml) and very high levels of plasma BDNF (higher than 58·32 ng/ml) using the basal levels as reference, in the whole samples of eating disorders, and in each of the subgroups (anorexia and bulimia).

Legends of Supplementary Figures

Figure S1. Genomic structure of the BDNF gene, according to all isoforms obtained from NCBI b35 assembly and described in the HapMap data. Haploview linkage disequilibrium plot for SNPs analyzed in samples of patients with eating disorders. Monomorfic SNPs and SNPs with Minor Allele Frequency less than 1% were excluded from the analysis and are not shown.

Figure S2: Box plot representing BDNF plasma levels for anorexia patients and for every SNP following the codominant, dominant, and recessive model. SNPs are ordered by genomic location of all genotypes for every SNP in the BDNF gene. SNPs represented in blue are the ones for which mean BDNF plasma levels are statistically different depending on the genotype. *Statistically significant after Bonferroni correction ($p<0.002$). A: major allele; a: minor allele.

Figure S3: Box plot representing BDNF plasma levels for bulimia patients and for every SNP following the codominant, dominant, and recessive model. SNPs are ordered by genomic location of all genotypes for every SNP in the BDNF gene. SNPs represented in blue are the ones for which mean BDNF plasma levels are statistically different depending on the genotype. *Statistically significant after Bonferroni correction ($p<0.002$). A: major allele; a: minor allele.

Figure S4: Box plot representing BDNF plasma levels for 50 unnaffected sib-pairs and for every SNP following the codominant, dominant, and recessive model. SNPs are ordered by genomic location of all genotypes for every SNP in the BDNF gene. SNPs represented in blue are the ones for which mean BDNF plasma levels are statistically different depending on the genotype. *Statistically significant after Bonferroni correction ($p<0.002$). A: major allele; a: minor allele.

Haplotype	rs7934165	270CT	Frequency	Family#	S	E (S)	Var (S)	Z	P
H1	A	C	0·46	22·9	17·55	24·14	6·22	-2·64	0·008*
H2	G	C	0·33	24·0	25·45	22·86	6·41	1·02	0·31
H3	G	T	0·21	22·4	18·55	14·14	5·45	1·89	0·06
H4	A	T	0·01	1·7	*****				

*Significant p value <0·05. #Number of informative families. S, Observed transmission of haplotypes to affected offspring. E, Expected transmission under Mendelian Inheritance. Var, Variance S-E. P, two-tailed p value.

Table 1: **BDNF haplotypes for anorexia nervosa, estimated from the two tag SNPs significantly overtransmitted or undertransmitted**

Haplotype	rs7124442	rs11030102	rs11030119	Frequency	Family#	S	E (S)	Var (S)	Z	P
H1	T	C	G	0·73	22·0	18·0	11·83	5·47	2·64	0·008*
H2	C	G	A	0·18	7·0	*****				
H3	C	C	A	0·04	0·0	*****				
H4	C	C	G	0·02	0·0	*****				
H5	C	G	G	0·01	0·0	*****				
H6	T	C	A	0·008	0·0	*****				
H7	T	G	A	0·007	0·0	*****				

*Significant p value <0.05. #Number of informative families. S, Observed transmission of haplotypes to affected offspring. E, Expected transmission under Mendelian Inheritance. Var, Variance S-E. P, two-tailed p value.

Table 2: **BDNF haplotypes for bulimia nervosa, estimated from the three tag SNPs significantly overtransmitted or undertransmitted**

Anorexia Nervosa	General Linear Model				
Genotypes	**Mean (95% CI)**	**R square**	**Beta**	**SE of Beta**	**P**
rs7934165A-270C carriers (n = 31)	49·0 (39·8-58·2)	0·03	3·3	7·8	0·6
Non rs7934165A-270C (n = 18)	45·7 (33·6-57·8)				

Bulimia Nervosa	General Linear Model				
Genotypes	**Mean (95% CI)**	**R square**	**Beta**	**SE of Beta**	**P**
rs7124442T-rs11030102C-rs11030119G carriers (n = 55)	43·6 (37·5-49·8)	0·11	20·6	10·0	0·02*
Non rs7124442T-rs11030102C-rs11030119G carriers (n = 6)	23·0 (4·4-41·6)				

AN patients carriers of rs7934165A and -270C haplotype undertransmitted in AN vs. non-carriers. BN patients carriers of rs7124442T, rs11030102C and rs11030119G haplotype found to be overtransmitted in BN patients vs. non carriers. P values based on BDNF plasma concentration.

Table 3: **Means, 95% CI, and general linear model of BDNF plasma levels according to the haplotypes associated to anorexia nervosa and bulimia nervosa**

Figure 1

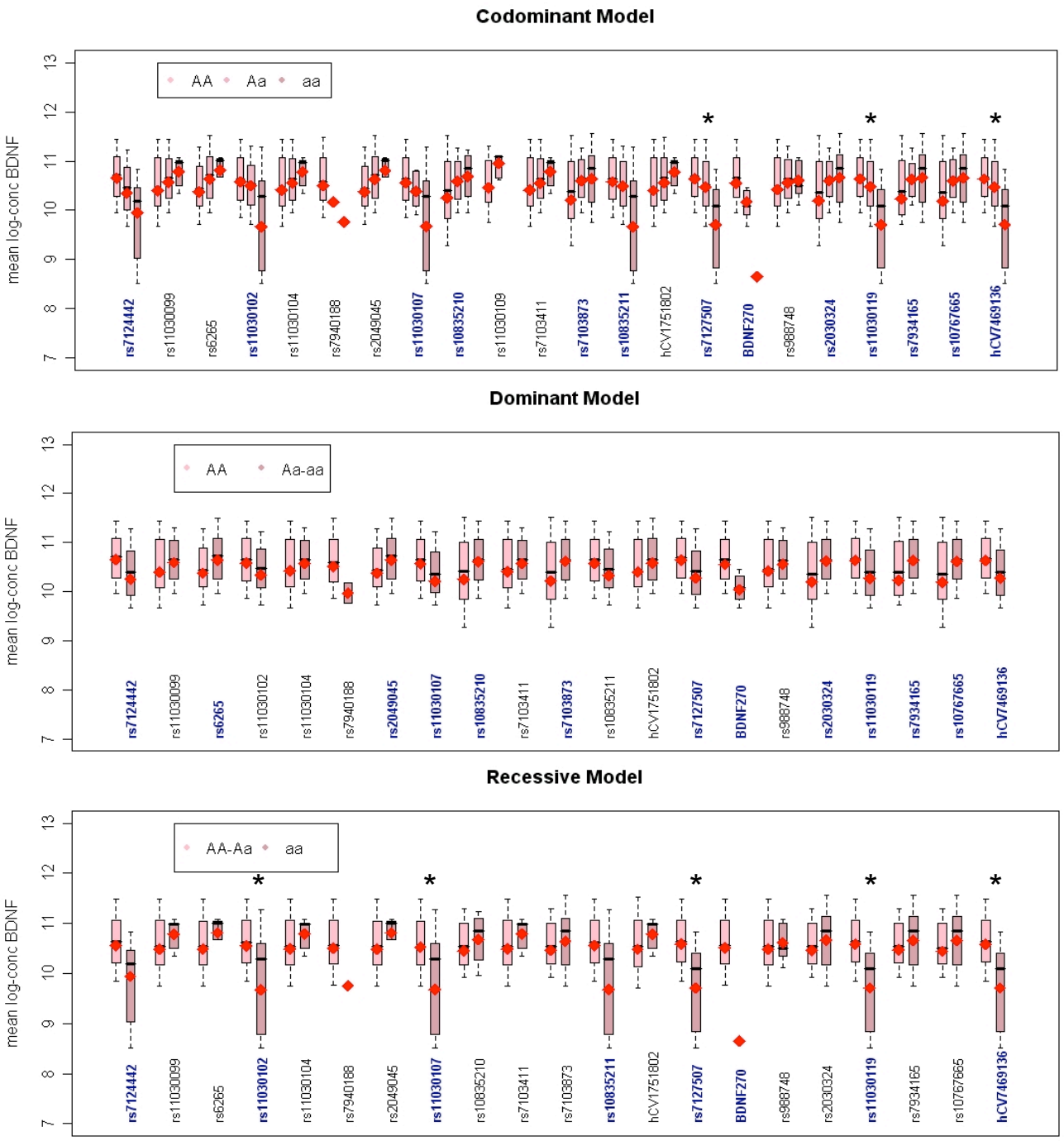
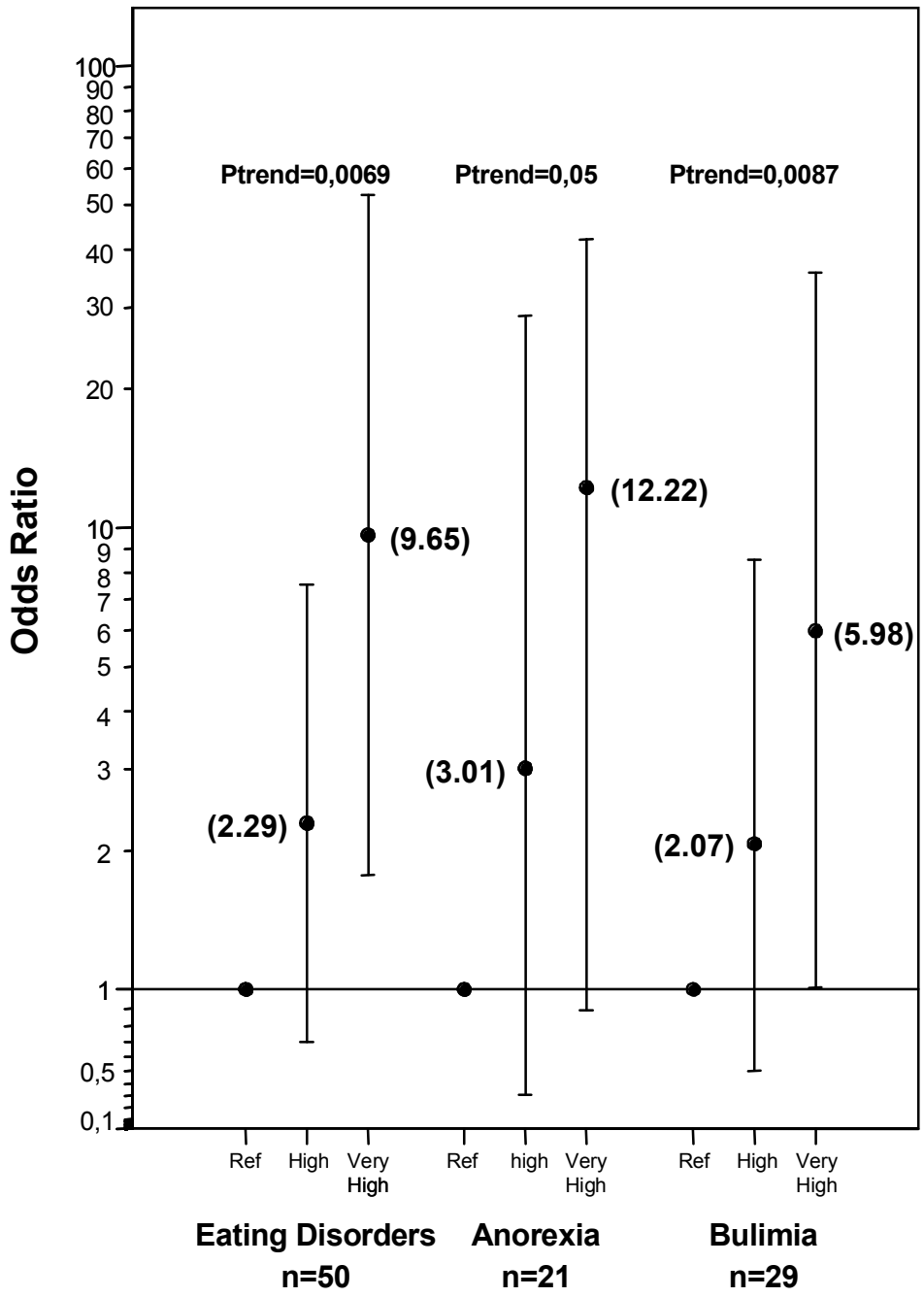

Codominant Model
AA
Aa
aa
mean log-conc BDNF
7
8
9
10
11
12
13
rs7124442
rs11030099
rs6265
rs11030102
rs11030104
rs7940188
rs2049045
rs11030107
rs10835210
rs11030109
rs7103411
rs7103873
rs10835211
hCV1751802
rs7127507
BDNF270
rs988748
rs2030324
rs11030119
rs7934165
rs10767665
hCV7469136
*
Dominant Model
AA
Aa-aa
mean log-conc BDNF
rs7124442
rs11030099
rs6265
rs11030102
rs11030104
rs7940188
rs2049045
rs11030107
rs10835210
rs7103411
rs7103873
rs10835211
hCV1751802
rs7127507
BDNF270
rs988748
rs2030324
rs11030119
rs7934165
rs10767665
hCV7469136
Recessive Model
AA-Aa
aa
mean log-conc BDNF
rs7124442
rs11030099
rs6265
rs11030102
rs11030104
rs7940188
rs2049045
rs11030107
rs10835210
rs7103411
rs7103873
rs10835211
hCV1751802
rs7127507
BDNF270
rs988748
rs2030324
rs11030119
rs7934165
rs10767665
hCV7469136
*

Figure 2


Odds Ratio
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0,5
0,1
Ptrend=0,0069
Ptrend=0,05
Ptrend=0,0087
(2.29)
(9.65)
(3.01)
(12.22)
(2.07)
(5.98)
Ref
High
Very High
Ref
high
Very High
Ref
High
Very High
Eating Disorders n=50
Anorexia n=21
Bulimia n=29

SNP Name	SNP set	Location	Function	Position	HW p value	% Geno	MAF
rs7124442	6	Exon 5	3'UTR	27633617	0·67	99·8	0·28
rs11030100	n·i·	Exon 5	3'UTR	27634159	1·0	79·8	0·0
rs11030099	1	Exon 5	3'UTR	27634162	0·36	99·0	0·24
rs2353512	n·i·	Exon 5	synonymous	27636238	1·0	99·8	0·0
rs6265	4	Exon5	Val/Met	27636492	0·11	99·2	0·22
rs3750934	n·i·	Intron	-	27636771	1·0	100·0	0·0
hCV9278624	n·i·	Intron	-	27637964	1·0	100·0	0·002
rs11030102	3	Intron	-	27638172	0·74	99·8	0·22
rs11030104	1	Intron	-	27641093	0·57	97·9	0·25
hCV26878171	n·i·	Intron	-	27645480	1·0	89·7	0·0
rs7940188	8	Intron	-	27650315	1·0	100·0	0·01
rs2049045	4	Intron	-	27650817	0·17	97·5	0·22
rs11030107	3	Intron	-	27651411	0·75	84·3	0·21
rs10835210	7	Intron	-	27652486	0·71	97·3	0·40
rs11030109	9	Intron	-	27653527	1·0	100·0	0·04
rs11030110	n·i·	Intron	-	27655823	1·0	86·0	0·0
rs7103411	1	Intron	-	27656701	0·51	97·5	0·25
rs7103873	2	Intron	-	27656893	0·42	99·2	0·46
rs10835211	3	Intron	-	27657941	0·99	99·0	0·21
hCV1751799	n·i·	Intron	-	27658097	1·0	99·8	0·0
hCV1751800	n·i·	Intron	-	27667224	1·0	99·8	0·002
hCV1751802	1	Intron	-	27667789	0·63	97·5	0·25
rs7127507	6	Intron	-	27671460	0·46	99·0	0·29
rs1967554	n·i·	Intron	-	27676135	1·0	100·0	0·0
rs4258332	n·i·	Intron	-	27676756	1·0	66·3	0·0
270CT	4	Exon 4	5'UTR	27678243	0·21	55·4	0·23
rs11030118	n·i·	Intron	-	27679639	1·0	99·4	0·0
rs988748	1	Exon 3	5'UTR	27681321	0·32	97·9	0·25
rs2030324	2	Intron	-	27683491	0·48	99·2	0·46
rs11030119	5	Intron	-	27684678	0·91	99·4	0·27
rs7937405	n·i·	Intron	-	27685895	1·0	100·0	0·0
rs11030120	n·i·	Intron	-	27687121	1·0	32·6	0·0
rs7934165	2	Intron	-	27688559	0·48	99·0	0·46
rs10767665	2	Intron	-	27690434	0·44	97·5	0·46
hCV7469136	5	Intron	-	27690996	0·83	98·8	0·27
rs10835216	n·i·	Intron	-	27697414	1·0	83·5	0·0

Tag SNPs capturing all BDNF SNPs with a minor allele frequency (MAF) higher than 1% are typed in bold. HWpval, Hardy Weinberg p value. SNPs with a MAF lower than 1% were excluded from the analysis. n.i.: Not included in the analysis. 5'UTR, 5' untranslated region. 3'UTR, 3' untranslated region.

Table S1: **Summary of SNPs analyzed and their position in relation to genome NCBI build 34, and the exons for all isoforms described in Hap Map**

Marker	Marker set	Genotype	Frequency	Families	S	E (S)	Var (S)	Z	P
rs7103411	1	CC	0·08	2	*****				
		CT	0·34	22	15·00	11·00	5·50	1·71	0·09
		TT	0·58	20	6·00	10·00	5·00	-1·79	0·07
rs7934165	2	AA	0·24	17	1·00	5·75	3·56	-2·52	0·01*
		AG	0·47	30	17·00	15·00	7·50	0·73	0·46
		GG	0·29	24	12·00	9·25	5·31	1·19	0·23
rs10767665		AA	0·24	15	1·00	5·25	3·19	-2·38	0·02*
		AG	0·48	26	15·00	13·00	6·50	0·78	0·43
		GG	0·28	20	10·00	7·75	4·44	1·07	0·29
rs2030324		CC	0·24	17	1·00	5·75	3·56	-2·52	0·01*
		CT	0·47	30	17·00	15·00	7·50	0·73	0·47
		TT	0·29	24	12·00	9·25	5·31	1·19	0·23
rs7103873		CC	0·24	16	1·00	5·25	3·31	-2·33	0·02*
		CG	0·47	30	17·00	15·00	7·50	0·73	0·48
		GG	0·29	25	12·00	9·75	5·56	0·95	0·34
rs11030102	3	CC	0·60	21	9·00	9·50	5·00	-0·22	0·82
		CG	0·37	24	11·00	12·00	6·00	-0·41	0·68
		GG	0·03	7	*****				
-270CT	4	CC	0·60	23	6·00	10·50	5·50	-1·92	0·06
		CT	0·36	24	17·00	12·00	6·00	2·04	0·04*
		TT	0·04	5	*****				
rs2049045		CC	0·04	3	*****				
		CG	0·35	22	14·00	11·00	5·50	1·28	0·21
		GG	0·61	21	7·00	10·00	5·13	-1·33	0·19
rs6265		AA	0·04	5	*****				
		AG	0·36	24	15·00	12·00	6·00	1·23	0·22
		GG	0·60	23	8·00	10·50	5·50	-1·07	0·29
rs11030119	5	AA	0·08	7	*****				
		AG	0·39	25	12·00	12·50	6·25	-0·20	0·84
		GG	0·53	21	9·00	9·75	5·06	-0·33	0·74
rs7124442	6	CC	0·08	9	*****				
		CT	0·40	27	13·00	13·50	6·75	-0·19	0·85
		TT	0·52	23	10·00	10·25	5·44	-0·11	0·92
rs10835210	7	AA	0·20	14	2·00	4·75	2·94	-161	0·11
		AC	0·45	28	15·00	14·00	7·00	0·38	0·71
		CC	0·35	23	11·00	9·25	5·19	0·77	0·44

SNPs with less than 10 informative families were excluded from the analysis. Tag SNPs are squared. S, observed transmission of genotype to affected offspring. E, expected transmission under Mendelian inheritance. Var, variance S-E. P, two-tailed P value. *significant P value <0·05. **Significant P value after Bonferroni correction (P <0·002). #Number of informative families. For marker sets with significant association for the Tag SNPs, the rest of the SNPs were analyzed.

Table S2: **Family based association study of BDNF SNPs in anorexia nervosa**

Marker	Marker set	Genotype	Frequency	Families	S	E (S)	Var (S)	Z	P
rs7103411	1	CC	0·08	7	*****				
		CT	0·34	25	14·00	12·50	6·25	0·60	0·55
		TT	0·58	22	9·00	10·50	5·38	-0·65	0·52
rs7934165	2	AA	0·24	18	8·00	7·75	4·19	0·12	0·90
		AG	0·47	33	17·00	16·50	8·25	0·17	0·86
		GG	0·29	20	8·00	8·75	4·69	-0·35	0·73
rs11030102	3	CC	0·60	21	15·00	9·75	5·06	2·33	0·02*
		CG	0·37	27	8·00	13·50	6·75	-2·12	0·03*
		GG	0·03	9	*****				
rs10835211		AA	0·03	9	*****				
		AG	0·36	27	8·00	13·50	6·75	-2·12	0·03*
		GG	0·61	22	15·00	10·00	5·25	2·18	0·03*
rs11030107		AA	0·63	19	14·00	8·75	4·56	2·46	0·01*
		AG	0·34	23	5·00	11·50	5·75	-2·71	0·007*
		GG	0·03	7	*****				
-270C/T	4	CC	0·60	20	8·00	8·75	4·69	-0·35	0·73
		CT	0·36	21	11·00	10·50	5·25	0·22	0·83
		TT	0·04	6	*****				
rs11030119	5	AA	0·08	9	*****				
		AG	0·39	25	6·00	12·50	6·25	-2·60	0·009
		GG	0·53	20	14·00	9·00	4·75	2·29	0·02*
hCV7469136		CC	0·08	8	*****				
		CT	0·38	24	6·00	12·00	6·00	-2·45	0·01*
		TT	0·54	20	14·00	9·00	4·75	2·29	0·02*
rs7124442	6	CC	0·08	7	*****				
		CT	0·40	27	5·00	13·50	6·75	-3·27	0·001**
		TT	0·52	23	18·00	10·75	5·56	3·07	0·002*
rs7127507		CC	0·09	6	*****				
		CT	0·40	24	6·00	12·00	6·00	-2·45	0·01*
		TT	0·51	20	16·00	9·50	4·87	2·94	0·003*
rs10835210	7	AA	0·20	14	7·00	5·75	3·19	0·7	0·48
		AC	0·45	29	15·00	14·50	7·25	0·19	0·85
		CC	0·35	20	7·00	8·75	4·69	-0·81	0·42

SNPs with less than 10 informative families were excluded from the analysis. Tag SNPs are squared. S, observed transmission of genotype to affected offspring. E, expected transmission under Mendelian inheritance. Var, variance S-E. P, two-tailed P value. *Significant p value <0·05. **Significant P value after Bonferroni correction (P <0·002). #Number of informative families. For marker sets with significant association for the Tag SNPs, the rest of the SNPs were analyzed.

Table S3: **Family based association study of BDNF SNPs in bulimia nervosa**

SNP	Codominant	Dominant	Recessive	Overdominant	Additive
rs7124442	0·003*	0·002*	0·01*	0·12	0·0006**
rs11030099	0·33	0·18	0·36	0·34	0·14
rs6265	0·13	0·05*	0·43	0·09	0·04*
rs11030102	0·005*	0·10	0·001**	0·99	0·01*
rs11030104	0·43	0·27	0·36	0·48	0·20
rs7940188	0·55	0·28	0·35	0·57	0·27
rs2049045	0·13	0·048*	0·43	0·09	0·04*
rs11030107	0·006*	0·032*	0·002**	0·63	0·004*
rs10835210	0·03*	0·01*	0·20	0·17	0·01
rs11030109	0·05	NA	NA	NA	0·05
rs7103411	0·41	0·26	0·36	0·46	0·19
rs7103411	0·41	0·26	0·36	0·46	0·19
rs7103873	0·02*	0·004*	0·30	0·08	0·01*
rs10835211	0·005*	0·09	0·001**	0·97	0·009*
hCV1751802	0·39	0·23	0·36	0·42	0·17
rs7127507	0·0001**	0·007*	$4{\cdot}7e^{-05}$**	0·77	0·0001**
-270C/T	0·005*	0·02*	0·006*	0·13	0·004*
rs988748	0·65	0·37	0·64	0·49	0·35
rs2030324	0·01*	0·002*	0·21	0·10	0·007*
rs11030119	0·0001**	0·006*	$4{\cdot}7e^{-05}$**	0·74	0·0001**
rs7934165	0·01*	0·004*	0·24	0·10	0·01*
rs10767665	0·01*	0·003*	0·19	0·12	0·007*
hCV7469136	0·0001**	0·006*	$4{\cdot}7e^{-05}$**	0·74	0·0001**

*Significant P value <0·05. **Significant P value after Bonferroni's correction (P value <0·002). P values based on BDNF concentration and adjusted by anorexia or bulimia status. NA, Non applicable. Monomorphic SNPs: rs11030100, rs2353512, rs3750934, hCV9278624, hCV26878171, rs11030110, hCV1751799, hCV1751800, rs1967554, rs11030118, rs7937405, rs11030120, and rs10835216. Tag SNPs used in the FBAT study are represented in bold.

Table S4: **P values for the comparison of BDNF plasma concentration depending on the genotype of SNPs within the BDNF gene for eating disorder patients**

SNP	Codominant	Dominant	Recessive	Overdominant	Additive
rs7124442	0·11	0·09	0·12	0·28	0·04*
rs11030102	0·91	0·68	0·79	0·76	0·66
rs11030107	0·81	0·51	0·82	0·57	0·53
rs10835210	0·007	0·002**	0·61	0·01*	0·03*
rs11030109	1·00	NA	NA	NA	0·90
rs7103873	0·01	0·004**	0·54	0·02*	0·04*
rs10835211	0·91	0·68	0·79	0·76	0·66
rs7127507	0·17	0·08	0·27	0·23	0·06
rs2030324	0·01	0·004**	0·40	0·04*	0·03*
rs11030119	0·16	0·07	0·27	0·21	0·05
rs7934165	0·01*	0·004**	0·40	0·04*	0·03*
rs10767665	0·007*	0·002**	0·30	0·04*	0·01*
hCV7469136	0·16	0·07	0·27	0·21	0·05

*Significant P value <0·05. **Significant P value after Bonferroni's correction (P <0·004). P values based on BDNF concentration and adjusted by case-control status. NA, Non applicable. Fifty unaffected sib pairs were used as controls for this analysis. Monomorphic SNPs: rs11030099, rs11030100, rs2353512, rs6265, rs3750934, hCV9278624, rs11030099, rs11030100, rs2353512, rs6265, rs3750934, hCV9278624, rs11030110, rs7103411, rs7103411, hCV1751799, hCV1751800, hCV1751802, rs1967554, BDNF270, rs11030118, rs988748, rs7937405, rs11030120, and rs10835216. Tag SNPs used in the FBAT study are represented in bold.

Table S5: **P values for the comparison of BDNF plasma concentration depending on the genotype of SNPs within the BDNF gene in 50 discordant sib-pairs for anorexia and bulimia**

Phenotype (n)	Mean Patient (95%CI)	Mean sibs (95%CI)	Mean paired differences (95%CI)	Paired t test p-value
ED (50)	52·7 (45·8-59·6)	41·0 (35·1-46·8)	11·8 (4·0-19·5)	0·004
AN (21)	57·8 (46·0-69·7)	42·6 (31·8-53·3)	15·3 (2·4-28·2)	0·023
BN (29)	49·1 (40·4-57·7)	39·8 (32·8-46·9)	9·2 (-1·0-19·4)	0·076

Table S6: **Means and 95% CI, and Mean paired differences and 95% CI of BDNF plasma levels in patients with eating disorders and the different clinical subtypes**

Figure S1

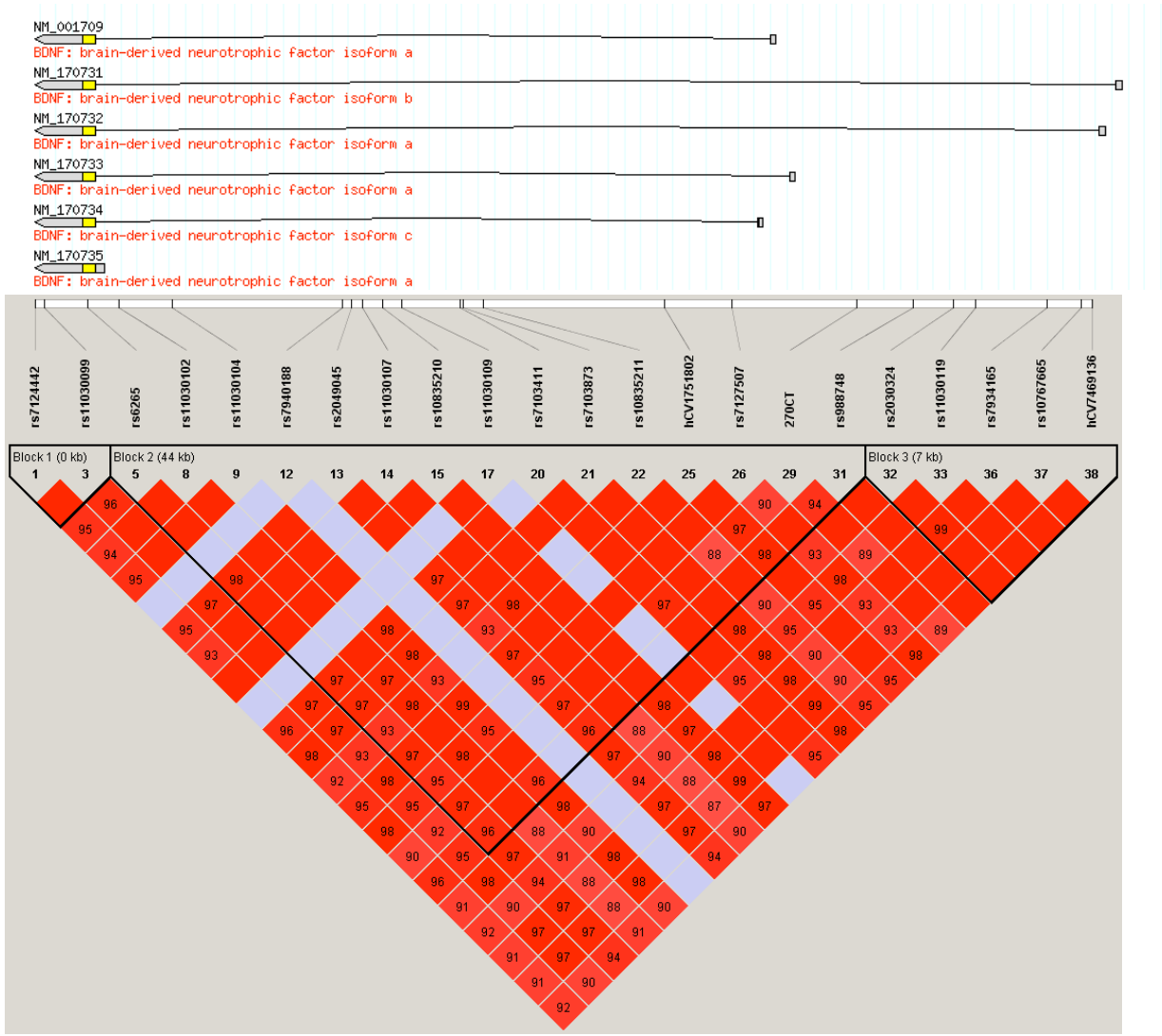

NM_001709
BDNF: brain-derived neurotrophic factor isoform a
NM_170731
BDNF: brain-derived neurotrophic factor isoform b
NM_170732
BDNF: brain-derived neurotrophic factor isoform a
NM_170733
BDNF: brain-derived neurotrophic factor isoform a
NM_170734
BDNF: brain-derived neurotrophic factor isoform c
NM_170735
BDNF: brain-derived neurotrophic factor isoform a
rs7124442
rs11030099
rs6265
rs11030102
rs11030104
rs7940188
rs2049045
rs11030107
rs10835210
rs11030109
rs7103411
rs7103873
rs10835211
hCV1751802
rs7127507
270CT
rs988748
rs2030324
rs11030119
rs7934165
rs10767665
hCV7469136
Block 1 (0 kb)
Block 2 (44 kb)
Block 3 (7 kb)

Figure S2

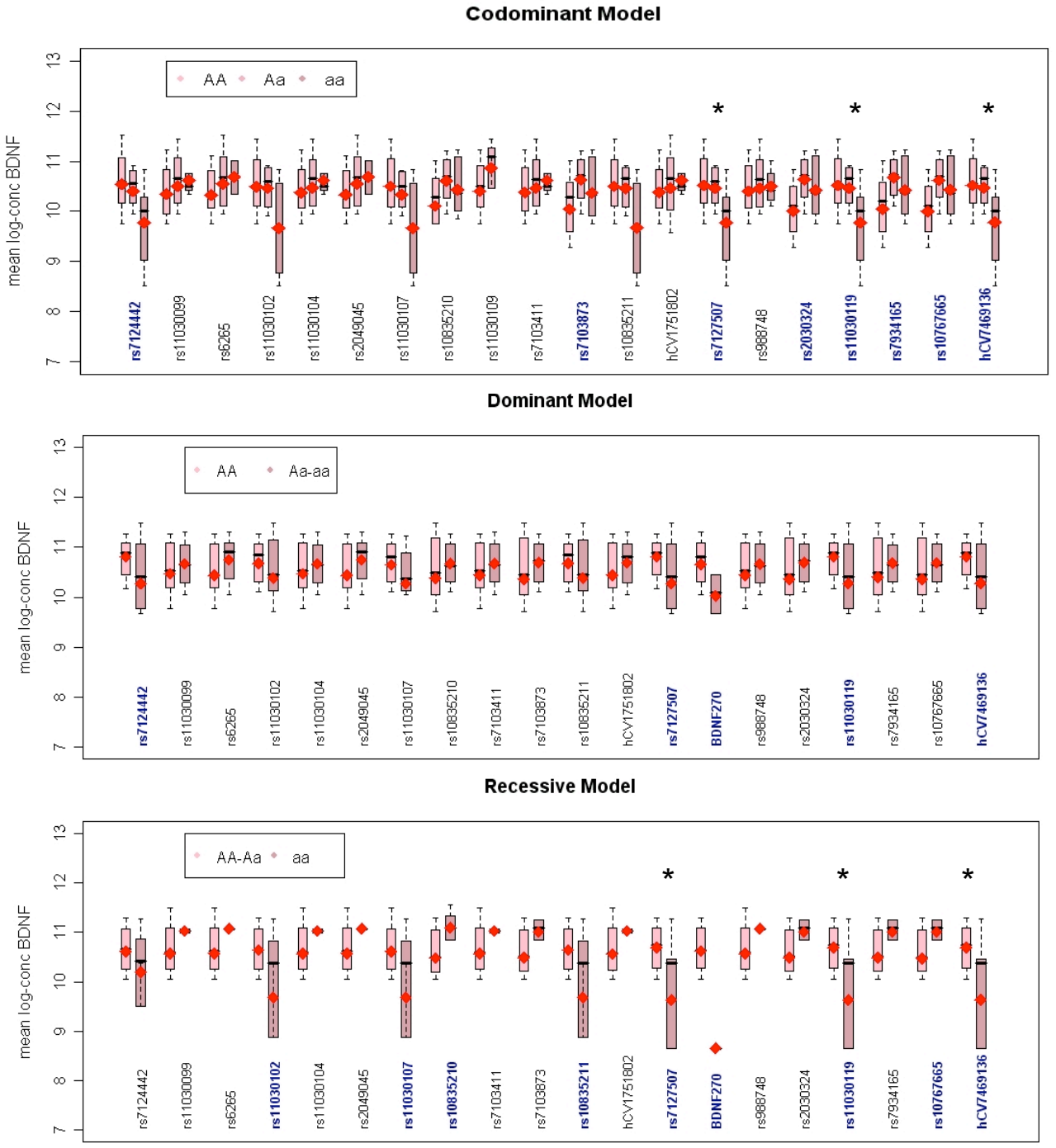

Codominant Model
AA
Aa
aa
mean log-conc BDNF
rs7124442
rs11030099
rs6265
rs11030102
rs11030104
rs2049045
rs11030107
rs10835210
rs11030109
rs7103411
rs7103873
rs10835211
hCV1751802
rs7127507
rs988748
rs2030324
rs11030119
rs7934165
rs10767665
hCV7469136
Dominant Model
AA
Aa-aa
BDNF270
Recessive Model
AA-Aa
aa

Figure S3

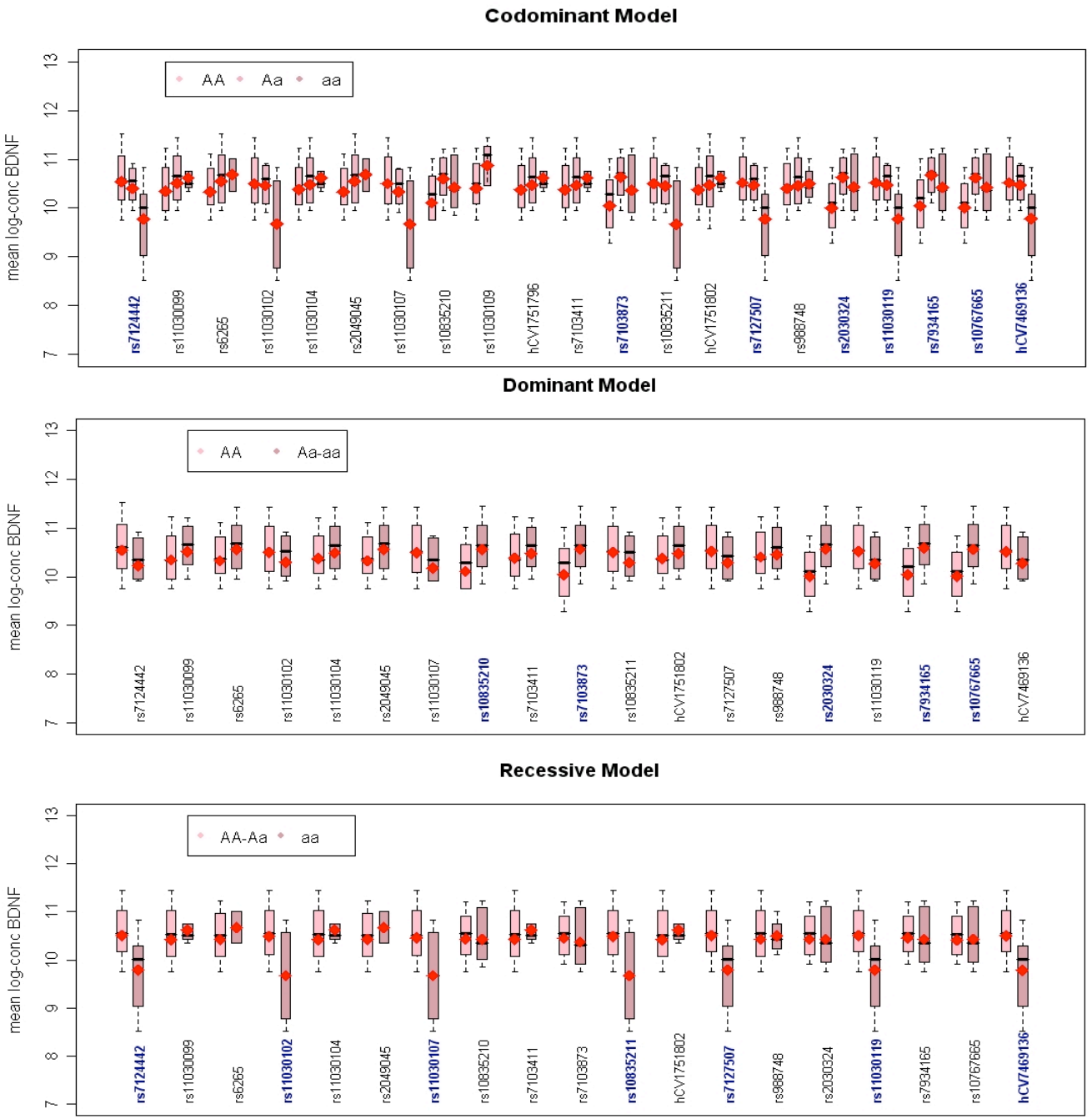
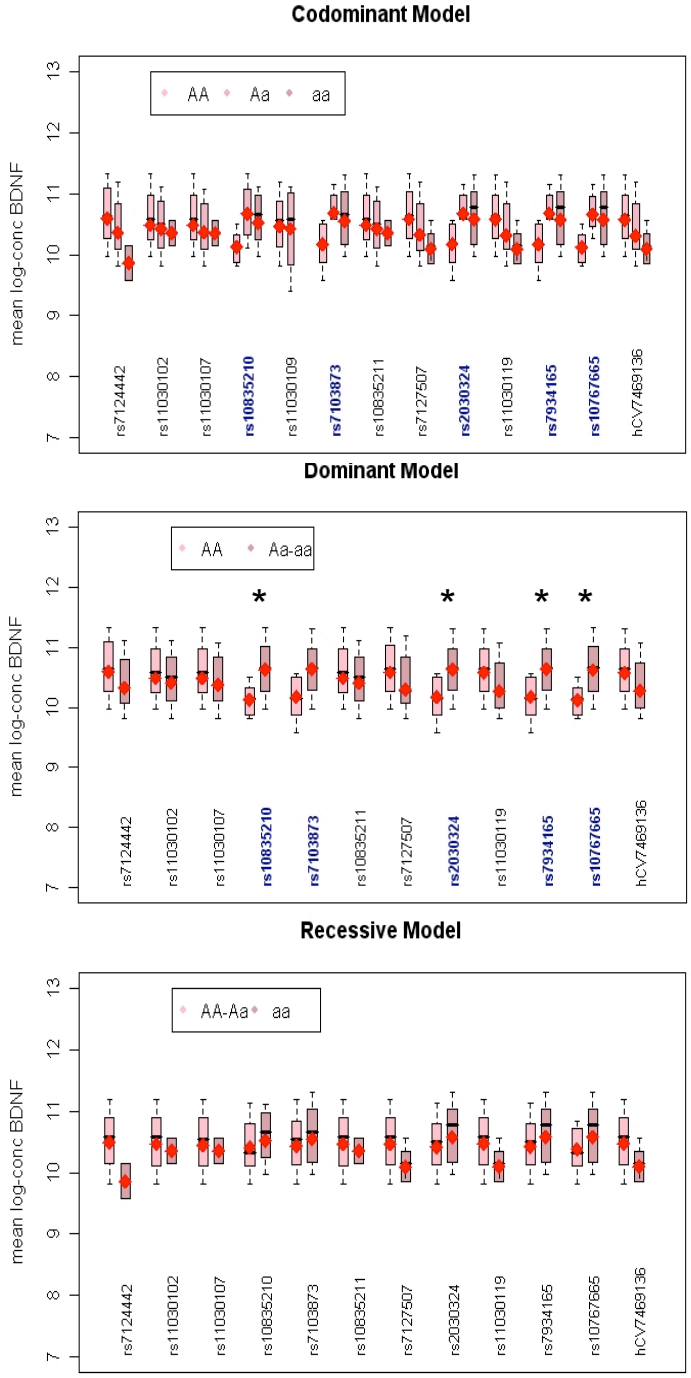

Codominant Model
AA
Aa
aa
mean log-conc BDNF
rs7124442
rs11030099
rs6265
rs11030102
rs11030104
rs2049045
rs11030107
rs10835210
rs11030109
hCV1751796
rs7103411
rs7103873
rs10835211
hCV1751802
rs7127507
rs988748
rs2030324
rs11030119
rs7934165
rs10767665
hCV7469136
Dominant Model
AA
Aa-aa
Recessive Model
AA-Aa
aa

Figure S4


Codominant Model
AA
Aa
aa
mean log-conc BDNF
rs7124442
rs11030102
rs11030107
rs10835210
rs11030109
rs7103873
rs10835211
rs7127507
rs2030324
rs11030119
rs7934165
rs10767665
hCV7469136
Dominant Model
AA
Aa-aa
Recessive Model
AA-Aa
aa