Genes, Parental Psychiatric Symptoms and Child Emotional Problems

Nurture versus Nature:
There and Back Again

Fleur Pieternel Velders

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Genes, Parental Psychiatric Symptoms and Child Emotional Problems

Nurture versus Nature: There and Back Again

Genen, psychiatrische symptomen van de ouders en emotionele problemen van het kind

opvoeding versus aanleg: daarheen en weer terug

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Chapter 1

Introduction

INTRODUCTION

Epidemiology

Worldwide, 10-20% of all children suffer from emotional and behavioural problems [1]. In preschool children, aggressive behaviour, hyperactivity and anxiety are already very prevalent [2]. Importantly, comorbidity of childhood disorders is high. Also, many of these disorders have sequelae that persist to adult life. This high comorbidity and the likelihood of a long-term vulnerability for mental health problems underscores the importance of a better understanding of the etiology of childhood disorders. Ultimately, we need to find answers to the question why some children get ill and others do not.

Parental psychiatric symptoms

Psychiatric symptoms of the parents place children at risk for the development of emotional and behavioural problems through various ways [3]. Parental mental health affects child development through genetic risk-transmission and by affecting the intrauterine environment of the developing fetus. After birth, the mental health of the parent will directly affect the interaction between the parent and the child. Also, psychiatric symptoms have been associated with the presence of various other stressors and may increase the risk of exposure to negative life events. In case of a major depression disorder (DSM IV) of the parent, the genetic risk transmission is estimated by a heritability of 37% [4]. Maternal depression during pregnancy has been associated with infant responses to novelty in the first 4 months of life, independent of postnatal maternal depression [5]. After birth, children of depressed mothers display more anxiety disorders, aggression, attention deficits, insecure attachment, poor self-esteem and poor peer relation [6]. Although relations of various parental psychological problems and family functioning with child development are well documented, the mechanisms underlying these prenatal and postnatal associations have not sufficiently been studied.

Parental psychopathology usually also affects the way of daily interaction within families. Hence in families with a depressed parent, the interaction between spouses is often characterized by increased hostility and tension [7]. So, children in these families are not only at an increased risk of emotional and behavioural problems, because they have a parent with depressive symptoms, but also due to an increased likelihood of exposure to hostility and poor family functioning. The debate remains to what extent these apparent risk factors independently contribute to child problem behaviour when analysed simultaneously. In this thesis, we examined the independent contributions of parental depression, parental hostility and family functioning to the risk of child problem behaviour. Also, we investigated whether genetic vulnerability of the child accounts for part of the individual vulnerability to the effects of parental psychiatric symptoms on child emotional and behavioural problems.

Genetic vulnerability: genetic main effects

Twin studies reported moderate to high heritability estimates for psychiatric disorders, however the actual genes accounting for these estimates remain to be determined. In this thesis, we aimed to identify genes related to psychiatric disorders using a genome wide association approach and a candidate gene approach.

Genome Wide Association Studies (GWAS)

GWAS are hypothesis free and examine the association between 2,500,000 single nucleotide polymorphisms (SNPs) across the human genome and a trait of interest. In contrast to candidate gene studies, GWAS are designed to discover genes that were not previously known to be related to the phenotype and hence may generate new hypotheses. In this thesis, we used the genome wide association approach to identify new genes related to cortisol secretion during the day and depressive symptoms. Unlike the other studies in this thesis that were embedded in the Generation R Study, this study was performed in the Rotterdam Study, a cohort of elderly people in the city of Rotterdam [8].

Also, we tested a new hypothesis based on the results of a GWAS on body mass index (BMI; weight (kg) /height $(m)^2$). In contrast to behavioural phenotypes, BMI is a biological measurement, which is easily obtained using standardized protocols and available in many studies. Furthermore, behaviour is likely related to a person's BMI [9]. Using a genome-wide approach, Frayling and colleagues identified an association between the fat and obesity-associated transcript gene (FTO) gene and overweight [10]. Following this initial discovery in 2007, SNPs in the FTO gene have consistently been associated with obesity and eating behaviour in adults and adolescents [11-13]. In light of evidence for high expression of FTO in the brain [14] and its relation with eating behaviour, the association of genetic variation in FTO with child behavioural phenotypes merits further investigation. Under the hypothesis that the effect of FTO on eating behaviour precedes the effect on BMI, we tested for an association between the FTO minor allele at rs9939609 and food approach, emotional control and symptoms of attention-deficit/ hyperactivity disorder (ADHD) in preschool children. Hence, we aimed to enhance gene discovery for psychiatric disorders by using GWAS findings of well-defined phenotypes that are likely related to behavioural phenotypes as well.

Candidate genes: HPA-axis

The hypothalamic-pituitary-adrenal (HPA-)axis is the main neuroendocrine system that is activated in response to stress. This axis consists of the hypothalamus, the anterior pituitary and the adrenal cortex. Glucocorticoids, i.e. cortisol, are the final effectors of the axis and exert a negative feedback effect on both corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) production and secretion in the hypothala-

mus and the pituitary dekloet endo rev 1998. In healthy persons, the diurnal secretion of cortisol is characterized by high levels in the morning followed by a decline in cortisol levels toward the evening. In response to various physical and psychological stressors, the HPA-axis becomes activated and as a result cortisol levels increase. To prepare for a successful active coping of the stressful situation, cortisol stimulates energy release by glucogenesis, lipolysis and increases the flow of oxygen and nutrition to the brain. Cortisol inhibits growth and the reproductive systems, and suppresses the immune system. Also, cortisol increases arousal and cognition, and suppresses feeding behaviour [15]. In healthy persons, this stress response is again terminated by activation of the negative feedback loop of the HPA-axis and homeostasis is restored. Hence, cortisol is essential for an adequate response to stress. Prolonged activation of the HPA-axis, however, may result in dysregulation of the stress system and psychopathology [16].

The effect of cortisol on target organs is mediated by two types of receptor: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). In the brain, MR mediates the onset of the stress response, whereas GR is involved in the termination of the stress response. Compared to MR, GR has less affinity for glucocorticoids and remains available at higher concentrations such as those occuring during exposure to psychosocial stressors [17]. Common genetic variation (SNPs) within the GR gene (GR, NR3C1) has been associated with hypersensitivity to cortisol, the occurrence of depression, and the response to antidepressant treatment [18]. The FK506 binding protein 5 (FKBP5), which acts as co-chaperone of GR, is associated with GR sensitivity to cortisol. SNPs within the FKBP5 gene have been associated with depression [19-20], treatment response and recurrence of depressive episodes [21]. HPA-axis activity is also under partial control of the serotonergic system. The serotonin transporter (5-HTT) plays a pivotal role in the duration and the intensity of the communication by serotonin with its receptors and postsynaptic targets. The function of 5-HTT is controlled by the polymorphic region in the promoter of the serotonin transporter gene (5-HTTLPR). The short allele of 5-HTTLPR has been associated with less serotonin binding in the brain, lower 5-HTT mRNA expression and lower serotonin reuptake compared to long allele carriers [22]. Animal and human studies showed that short allele carriers may be more reactive to stress [23-25].

Genetic vulnerability: gene-environment interaction

In light of their inherent complexity, it seems likely that psychiatric disorders are caused by small effects of many genes in interaction with other genes and in interaction with the environment [26]. It has been posited that the missing heritability of psychiatric disorders may partly be hidden in epistasis, epigenetics, gene-environment correlation or gene-environment interactions [27]. In this latter framework, the effect of an environmental risk factor differs according to a person's genotype, or the genetic effect on the phenotype may depend on certain environmental circumstances. In this thesis,

we studied the interaction between genes involved in (HPA)-axis functioning and parental psychiatric problems on the risk for child emotional and behavioural problems in preschool children. Accordingly, we investigated the interaction effect between SNPs located in the GR and FKBP5 gene region with child attachment on child cortisol reactivity. Also, we studied the possible interaction between these candidate SNPs and maternal psychiatric symptoms on child emotional problems. Last, we examined the possible interaction between 5-HTTLPR and prenatal and postnatal maternal anxiety and its effect on child emotional development.

Setting: The Generation R Study

The Generation R Study is a prospective population-based cohort study from fetal life onwards in the city of Rotterdam, the Netherlands. It was designed to identify early biological and environmental determinants of growth, development and health in fetal life and childhood [28-29]. In short, all pregnant women living in the study area with a delivery date between April 2002 and January 2006 were eligible for enrollment in the Generation R Study. In total, 9,778 pregnant women were included, of whom 8,880 enrolled in the prenatal part of the study. The participating women gave birth to 9,745 live born children. Due to exclusion of participants in the pilot phase (12%) and because of withdrawal from the study (7%), 7,893 children participated in the postnatal phase of the Generation R Study.

Aim of this thesis

The main aim of this thesis was to study the effect of common genetic variation, parental psychiatric symptoms, and gene-environment interaction effects during pregnancy and early childhood on the risk of emotional and behavioural problems in preschoolers.

In *chapter 2*, we examined the impact of prenatal and postnatal psychiatric symptoms and family function of both mothers and fathers on the risk for child emotional and behavioural problems. In *chapter 3*, we focus on genetic main effects on child emotional and behavioural problems. First, we study the association between a candidate SNP in the FTO gene and child eating behaviour, symptoms of ADHD and self control in preschool children (*chapter 3.1*). In *chapter 3.2*, we sought to identify new genes related to both cortisol secretion and depression using a GWAS approach and a candidate gene approach. *Chapter 4* describes our results on gene-environment interaction with candidate SNPs in the GR gene and the FKBP5 gene. In *chapter 5*, we examined the interaction between 5-HTTLPR and prenatal maternal chronic difficulties and anxiety symptoms and postnatal maternal anxiety symptoms on child emotional problems. In *chapter 6*, the main findings of these studies are reviewed, methodological considerations and clinical implications are discussed and we reflect on future perspectives.

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Chapter 2

Parental psychiatric problems during pregnancy and early childhood

Chapter 2.1

Prenatal and postnatal psychological symptoms of parents and family functioning: the impact on child emotional and behavioural problems.

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ABSTRACT

Background: Although relations of various parental psychological problems and family functioning with child development are well documented, it remains unclear whether specific prenatal or specific postnatal risk factors are independently associated with child emotional and behavioural problems, or whether observed associations can be explained by general parental psychopathology. Using a stepwise approach, we examined the effects of prenatal and postnatal parental depressive symptoms, prenatal and postnatal hostility of the parents, as well as prenatal family functioning on the risk of child emotional and behavioural problems.

Methods: This study was embedded in Generation R: a population-based cohort from fetal life onwards. Mothers and fathers of 2698 children provided information about depressive symptoms, symptoms of hostility and family functioning during pregnancy and 3 years after birth. Mother and father each reported on child behaviour when the child was 3 years old.

Results: Parental depressive symptoms increased the risk of child emotional and behavioural problems, but this increase was explained by postnatal parental hostile behaviour. Postnatal symptoms of hostility of mothers (OR = 1.34, *p* value <0.001) and postnatal symptoms of hostility of fathers (OR = 1.30, *p* value <0.001) each contributed independently to the risk of child emotional and behavioural problems.

Conclusions: Postnatal parental hostility is associated with an increased risk of child emotional and behavioural problems, independent of parental depressive symptoms. These findings suggest that prevention and intervention strategies should focus on psychological symptoms of both mothers and fathers, in particular on hostile behaviour, in families with young children.

INTRODUCTION

A broad range of psychological problems of parents places children at risk for the development of emotional and behavioural problems. A key example is the effect of maternal depression on child development. Not only is depression in children of depressed mothers more frequent and more severe than in children of non-depressed mothers, but these children also display more anxiety disorders, aggression, attention deficits, insecure attachment, poor self-esteem and poor peer relations [1-2]. The relation between psychopathology of parents and child development is not limited to the mother-child relationship [3-4]. For instance, postnatal paternal depression was associated with a higher likelihood of a psychiatric diagnosis in children at the age of 7 [5]. Next to the evidence for a postnatal effect of parental psychopathology on child development, there are also several reports suggesting a direct relation between maternal stress during pregnancy, such as depression and anxiety, and child development [6]. Interestingly, father s' prenatal depression has also been associated with child development such as excessive infant crying [7], child anxiety [8], and conduct problems [9]. Genetic effects, programming effects in utero and differentiation effects after birth may account for these prenatal and postnatal associations [10]. During pregnancy, the comparison of the effect of maternal risk factors on the likelihood of child internalizing problems with the effect of paternal risk factors has been used to investigate the causality of the underlying association [11]. If only a maternal prenatal relation is found, this may be the result of specific intra-uterine programming effects. A prenatal effect of paternal risk factors more likely reflects long lasting effects such as a genetic risk for psychopathology or residual confounding (i.e. unmeasured variables account for the association).

Like depression, parental hostility is also a significant threat to child development [12-13]. Hostile behaviour of mothers and fathers is related to less optimal interactions with their children [14]. Parent-child hostility gives rise to fear, anger and distress, and increases the likelihood of aggressive behaviour and anxiety of the child [14-15]. The actual effect of parental hostility seems to depend mainly on emotional and cognitive processes within the child, and on family processes such as the level of involvement of the child in parental disputes [16].

By way of daily interaction within families, parental psychopathology usually also affects contextual factors. In families with a depressed parent, the interaction between spouses is often characterized by increased hostility and tension [17]. These families report poor family functioning more frequently than families with no depressed parents [18]. Therefore, children in these families are not only at an increased risk of emotional and behavioural problems, because they have a parent with psychological problems, but also due to an increased likelihood of exposure to marital conflict and poor family functioning.

Previous research mostly focused on the interrelation of parental depression, hostility, marital conflict and family functioning on child development, and mediators of these associations [16, 19-21]. In a recent review in this field, it was suggested that parental psychopathology and family functioning have reciprocal effects without a causal primacy of one of the two [21]. The debate remains to what extent these apparent risk factors independently contribute to child problem behaviour when analysed simultaneously. Insight in the independent contributions of parental depression, parental hostility and family functioning to the risk of child problem behaviour is important for the development of effective prevention and intervention strategies.

In the present study, we aimed to test the following hypotheses, 1) prenatal psychological symptoms of parents are a risk factor for child emotional and behavioural problems independent of postnatal parental symptoms, 2) parental depressive symptoms and parental symptoms of hostility each contribute to the risk of emotional and behavioural problems in children. Given the dyadic nature of family functioning, we also expected to find an additional effect of family functioning on the risk of child problems, and 3) any prenatal effect of maternal psychological symptoms exceeds the effect of paternal psychological symptoms during pregnancy, due to direct physiologic effects via the mother on the intrauterine environment of the fetus.

We tested these hypotheses in 2698 families participating in an ongoing population-based cohort. To explore the specificity of our findings, we also examined the effect of other parental psychological symptoms, such as psychoticism and anxiety, on child emotional and behavioural problems. Furthermore, we examined the effect of parental depressive symptoms, parental hostility, and family functioning on subtypes of emotional and behavioural problems; emotionally reactive behaviour, anxious/depressed behaviour, somatic complaints, withdrawn behaviour, attention problems and aggressive behaviour.

METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from foetal life onwards in Rotterdam, the Netherlands. The Generation R Study has previously been described in detail [22]. All children were born between April 2002 and January 2006. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam (numbers: prenatal, MEC 198.782/2001/31 and postnatal, MEC 217.595/2002/202). Written informed consent was obtained from all adult participants.

Population of analysis

In the Generation R cohort, complete information on depressive symptoms, symptoms of hostility during pregnancy and family functioning was obtained from 3425 mothers and fathers. Families without father participation were not included in this study. Of the 3425 couples, 2698 filled out the questionnaire about child behaviour. Hence in total, 2698 couples and children (79% of 3425) were included in the analyses.

Prenatal and postnatal psychological symptoms of the parents

Psychological symptoms of the parents were assessed at 20 weeks of pregnancy and when the child was 3 years old with the Brief Symptom Inventory (BSI), a validated self-report questionnaire with 53 items to be answered on a five-point scale, ranging from "0=not at all" to "4=extremely" [23-24]. These 53 items are classified in eight subscales; Depression, Hostility, Anxiety, Phobic Anxiety, Paranoid Ideation, Psychoticism, Interpersonal Sensitivity and Obsessive-Compulsive. This study focused mainly on depression and hostility. The Depression scale consists of 6 items e.g. "I am feeling suicidal" and "I have no interest in anything". The Hostility scale consists of 5 items e.g. "I have an urge to hit, injure or cause pain to others" and "I often get involved in arguments". Higher scores on these scales represent an increased occurrence of depressive symptoms or symptoms of hostility.

Prenatal family functioning

Family functioning was assessed by the subscale General Functioning of the Family Assessment Device [25] at 20 weeks pregnancy. General Functioning is a validated overall self-report measure of health and pathology of a family and consists of 12 items. Half of the items describe healthy functioning, e.g. "In times of crisis, we can turn to each other for support". The other half describes unhealthy items, e.g. "There are a lot of unpleasant and painful feelings in our family." Parents were asked to rate how well each item described their family by selecting from four different responses: strongly agree, agree, disagree or strongly disagree. The scores per item were summed and divided by 12 yielding a total score from 1 to 4. A higher total score translates into less well functioning families or poor family functioning.

Child behaviour

The Child Behavior Checklist/1½-5 (CBCL/1½-5) was used to obtain standardized parent reports of children's problem behaviour at the age of 3 years. This behavioural questionnaire contains 99 items, which are scored on a three-point scale; 0 = not true, 1 = somewhat true or sometimes true and 2 = very or often true, based on the two preceding months. The Total Problems score is obtained by summing the scores of all 99 items. The Internalizing scale score is a sum score of the items (N=36) in four syndrome scales: Emotionally Reactive, Anxious/Depressed, Somatic Complaints, and Withdrawn. The

Externalizing scale score is a sum score of the items (N=24) in the Attention Problems and Aggressive Behavior syndrome scales. The psychometric properties of the CBCL are well established [26]. In this study, we used the broadband scale Internalizing scale, the Externalizing scale and the syndrome scales (emotionally reactive, anxious/depressed, somatic complaints, withdrawn, attention problems, and aggression). The data could not be normalized and were analyzed as dichotomized variables. To obtain a score on emotional and behavioural problems based on the report of both parents, the scores on Internalizing and Externalizing of mother and father were standardized (Z-scores) and averaged. If only the score of one parent was available, this score was used (12%). Dutch norm scores have not been published. As in previous analyses, we defined a non-optimal score as the highest 20 percent of Internalizing and Externalizing item scores [27]. Likewise we calculated non-optimal scores of the syndrome scales.

Other measurements

Information on infant birth weight and gender were obtained from midwife and hospital registries. Gestational age was established by foetal ultrasound examinations within the Generation R Study. Information on parental age at child birth, parental educational level, maternal smoking and maternal alcohol use during pregnancy, birth order, age of the infant, and ethnicity of the infant was obtained by questionnaire. The highest completed education (primary school, secondary school and higher education) determined the educational level of the parents. Ethnicity of the infant was classified into two categories based on the parental country of birth. If both parents were non-Dutch, mother's ethnicity determined the ethnicity of the child. The group of children classified as Western includes Dutch, American Western (non-Hispanic Whites), Asian Western (Japan), European and Australian children. The Non-Western group is comprised of children with a Turkish, Moroccan, Surinamese, Cape Verdean, Dutch Antillean, African, American non-Western (Afro-Americans, Hispanics) and Asian non-Western (Asia except Japan) ethnicity [28-29]. Maternal smoking and maternal alcohol use were assessed at three time-points during pregnancy and categorized into "yes, during pregnancy" and "never during pregnancy". The inclusion of these potential confounders was determined a priori and based on existing knowledge about the association between parental psychopathology and child behaviour [6].

Statistical analysis

In a non-response analysis, differences in baseline characteristics of responders (n=2698) and non-responders (n=727) on the assessment of child behaviour were compared with the Chi-square statistic for categorical variables, the independent *t* test for normally distributed continuous variables and the Mann-Whitney *U* test for non-normally distributed continuous variables. Likewise, we compared baseline characteristics between children with low scores on emotional and behavioural problems and children with high scores

on emotional and behavioural problems. The correlation between the determinants was analyzed using the Spearman's correlation coefficient for non-parametric variables (ρ_{sp}). The continuous measures of parental depressive symptoms, family functioning and symptoms of hostility were expressed per standard deviation to facilitate comparisons of effect sizes.

First, we examined the direct effect of prenatal depressive symptoms, prenatal hostility symptoms, prenatal family functioning, postnatal depressive symptoms and postnatal hostility symptoms of the parents on child internalizing problems and child externalizing problems. These multivariate linear regression analyses were adjusted for covariates, but not for the other determinants. To test the specificity of these associations for depressive symptoms and hostility, we additionally examined the association of other prenatal maternal BSI subscales (Phobic Anxiety, Psychoticism, Paranoid Ideation, Obsessive-Compulsive, Interpersonal Sensitivity, Somatization and Anxiety) with child internalizing and externalizing problems.

Second, we examined the independent contribution of parental psychological problems and family functioning to the development of child internalizing and externalizing problems using multivariate linear regression analyses in five successive models. The first three models included the prenatal determinants, whereas the postnatal determinants were added in model 4 and model 5. To test for significant difference between the odds ratios, we produced 84% confidence intervals (84%CI) around the odds ratios and examined the overlap. As reported by Julious, the level of statistical significance between the two groups would be 5% or lower if the 84% confidence intervals around the odds ratios do not overlap [29]. Third, we examined the association of parental psychological symptoms and family functioning with subtypes of internalizing and externalizing problems (model 5).

All analyses were controlled for child gender, birth weight, birth order, child ethnicity, child age, maternal smoking and drinking behaviour during pregnancy, parental age, as well as for parental education. Missing data were imputed using multiple imputation procedures. Test statistics and regression coefficients were averaged across five imputed data sets. The level of significance for all analyses was set at $\alpha = .05$. All statistical analyses were carried out using PASW Statistics, version 17.0 for Windows [30].

Non-response analysis

Mothers who did not complete the CBCL/1½-5 were on average younger at child birth (29.2 vs. 31.6 years, $t = 12.27$, $p < 0.001$), were less likely high educated (20.3 vs. 37.5%, $\chi 2 = 76.17$(1df), $p < 0.001$), and more likely to smoke during pregnancy (26.2 vs. 18.5%, $\chi 2 = 21.06$(1df), $p < 0.001$ than responding mothers. Likewise, fathers who did not complete the CBCL/1½-5 were on average younger at child birth (32.2 vs. 33.9 years, $t = 6.598$, $p < 0.001$), and were less high educated (26.5 vs. 40.1%, $\chi 2 = 45.67$(1df), $p < 0.001$) than

responding fathers. Children of non-responding mothers had on average a lower birth weight (3363 vs. 3480 g, $t = 5.05$, $p < 0.001$) and the origin of these children was less likely Dutch or Western (63.6 vs. 84.8%, $\chi 2 = 163.16(1df)$, $p < 0.001$) compared with children of mothers who did complete the CBCL/1½-5 .

RESULTS

Table 1 presents the subject characteristics in the group of children with low internalizing problems compared with the subject characteristics in the group of children with high internalizing problems. Mothers of children with high internalizing problems were on average younger at child birth (mean 30.8 vs. 31.8 years, $t = 5.39$, $p < 0.001$) and were less likely highly educated (33.8 vs. 39.2%, $\chi 2 = 5.06(1df)$, $p = 0.021$) than mothers of children with low internalizing problems. Paternal characteristics showed a similar distribution over the groups compared with maternal characteristics. The children with high internalizing problems were more likely firstborns (72.8 vs. 60.5%, $\chi 2 = 27.88(1df)$, $p < 0.001$), had on average a lower birth weight (3422 vs. 3495 g, $t = 2.27$, $p = 0.006$) and were less often of Western origin (78.3 vs. 86.9%, $\chi 2 = 19.71(1df)$, $p < 0.001$) compared with children with low internalizing problem scores. The comparison of children with low externalizing problems and high externalizing problems showed a similar distribution of characteristics, except for maternal smoking during pregnancy (never smoked during pregnancy 82.9 vs. 76.0%, $\chi 2 = 13.96(1df)$, $p < 0.001$) and gender (boys 47.1 vs. 60.3%, $\chi 2 = 29.74(1df)$, $p < 0.001$) (data not shown).

Prenatal depressive symptoms, prenatal hostility, prenatal family functioning, postnatal depressive symptoms and postnatal hostility of the parents were significantly correlated (p value <0.001), and the highest correlation was found between depressive symptoms and symptoms of hostility (e.g. ρ_{sp} prenatal maternal depressive symptoms - prenatal maternal hostility 0.50, p value <0.001) (Supplementary material Table 1). Colinearity among BSI sub-scales was indicated by correlations of 0.31-0.64. For instance, we found a correlation of 0.52 for BSI depression and BSI anxiety, for BSI depression with BSI phobic anxiety a correlation of 0.37, for BSI hostility with BSI anxiety a correlation of 0.48 and a correlation for hostility with phobic anxiety of 0.31.

As presented in table 2, parental prenatal depressive symptoms, parental prenatal symptoms of hostility, parental prenatal family functioning, parental postnatal depressive symptom and parental postnatal symptoms of hostility were all significantly associated with an increased likelihood of child internalizing problems (OR range 1.14-1.54, p values <0.01) and child externalizing problems (OR range 1.06-1.38, p values <0.01). The other prenatal BSI subscale scores of the mother also predicted child internalizing problems (OR of Phobic Anxiety 1.18, OR of Paranoid Ideation 1.24, OR of Psychoticism 1.26, OR of Interpersonal Sensitivity 1.30, OR of Anxiety 1.34, OR of Obsessive-Compulsive

Table 1 Subject characteristics (n=2698).

	Child Internalizing Problems reported by both parents			
	Low internalizing problems[a]	**High internalizing problems[a]**		
	(n=2162)	(n=536)	test statistic[c]	*p*-value
Mother				
Age at child birth (years)	31.8(4.0)	30.8(4.1)	5.39	<0.001
Education (%)				
High	39.2	33.8	5.06	0.02
Smoking during pregnancy (%)				
Never	82.0	79.9	1.27	0.26
Alcohol use during pregnancy (%)				
Never	30.8	37.1	8.00	0.005
Birth Order (%)				
First child	60.5	72.8	27.88	<0.001
Father				
Age at child birth (years.)	34.0 (5.0)	33.3(4.8)	2.88	0.004
Education (%)				
High	41.6	36.2	5.17	0.02
Child				
Gender (% boys)	50.1	48.3	0.57	0.45
Gestational age at birth (weeks)	40.1(27.6-43.4)[b]	40.1(29.6-42.9)[b]	1.44	0.21
Birth weight (gram)	3495(553)	3422(583)	2.27	0.01
Ethnicity (%)				
Dutch/Other Western	86.9	78.3	19.71	<0.001

Low internalizing problems were defined as scores below the 80th percentile on the Internalizing scale of the Child Behavior Checklist and High internalizing problems as scores at the 80th percentile and higher on the Internalizing scale of the Child Behavior Checklist.

[a]mean (standard deviation) unless otherwise indicated

[b]median (100% range)

[c]with the chi-square statistic for categorical variables (parental education, smoking during pregnancy, alcohol during pregnancy, gender, child ethnicity), the independent t-test for normally distributed continuous variables (parental age, birth weight) and the Mann Whitney U test for non-normally distributed continuous variables (gestational age).

1.37, all *p* values <0.001) and child externalizing problems (OR of Phobic Anxiety 1.15, OR of Paranoid Ideation 1.23, OR of Psychoticism 1.21, OR of Interpersonal Sensitivity 1.27, OR of Anxiety 1.22, OR of Obsessive-Compulsive 1.42, all *p* values <0.01).

In table 3, we present the association of parental psychological symptoms and family functioning with child internalizing problems in five successive models. The prenatal analyses showed that prenatal depressive symptoms of mothers and fathers each predicted internalizing problems (model 1). However, these associations were no longer

Table 2 The associations of parental symptoms of psychopathology and prenatal family functioning with child emotional and behavioural problems as reported by both parents.

	Child Internalizing Problems at 3 years reported by both parents (per sd)			Child Externalizing Problems at 3 years reported by both parents (per sd)		
	OR[a]	95% CI	*p* value	OR[a]	95% CI	*p* value
Prenatal depressive symptoms per sd (n=2698)						
Mother	1.21	1.11;1.32	<0.001	1.23	1.13;1.34	< 0.001
Father	1.18	1.08;1.29	<0.001	1.16	1.060;1.26	0.001
Prenatal hostility symptoms per sd (n=2698)						
Mother	1.27	1.16;1.39	<0.001	1.29	1.18;1.41	<0.001
Father	1.23	1.13;1.34	<0.001	1.23	1.13;1.34	<0.001
Prenatal family functioning per sd (n=2698)						
Mother	1.14	1.04;1.25	<0.001	1.24	1.13;1.36	<0.001
Father	1.18	1.07;1.30	0.001	1.19	1.08;1.31	0.001
Postnatal depressive symptoms per sd (3years after birth)(n=2692)						
Mother	1.37	1.25;1.50	<0.001	1.32	1.21;1.44	<0.001
Father	1.24	1.14;1.35	<0.001	1.26	1.16;1.37	<0.001
Postnatal hostility symptoms per sd (3years after birth)(n=2696)						
Mother	1.54	1.40;1.69	<0.001	1.51	1.38;1.66	<0.001
Father	1.42	1.30;1.55	<0.001	1.44	1.32;1.57	<0.001

Reference group = children with Internalizing Problem scores/ Externalizing Problem scores below the 80th percentile on the Child Behavior Checklist.
All analyses were adjusted for child gender, birth weight, birth order, ethnicity, child age at questionnaire, maternal smoking and alcohol use during pregnancy, parental age and parental educational level. Small differences in numbers due to the exclusion of outliers in postnatal psychological symptoms.
Abbreviations: OR; odds ratio, CI; confidence interval

[a]OR's represent the increased risk of internalizing and externalizing problem scores per standard deviation (sd) increase of the determinants.

significant when prenatal parental hostility was added to the regression analysis (model 2). In model 3, a higher score on prenatal family functioning experienced by the mother was associated with an increased risk of child internalizing problems (OR 1.25, 95%CI 1.12; 1.39, *p* value <0.001). Prenatal family functioning experienced by the father was not significantly associated with child internalizing problems. In model 4 and 5 postnatal determinants were added to the analyses. As shown in these models, we found that the initially significant associations of postnatal depressive symptoms of mother and father with an increased risk of internalizing problems were no longer significant after adding postnatal parental symptoms of hostility. The final model (model 5) showed that poor family functioning experienced by the mother during pregnancy (OR 1.23, 95%CI 1.10; 1.37, *p* value <0.001), postnatal symptoms of hostility of mother (OR1.35, 95%CI 1.20;

Table 3 The association of family functioning and parental psychopathology with child internalizing problems as reported by both parents in mutually adjusted successive models.

	Child Internalizing Problems at 3 years reported by both parents (per sd)													
Successive	model 1 (n=2698)			model 2 (n=2698)			model 3 (n=2698)			model 4 (n=2687)			model 5[a] (n=2685)		
models	OR[b]	95% CI	*P*	OR[b]	95% CI	*P*	OR[b]	95% CI	*p*	OR[b]	95% CI	*p*	OR[b]	95% CI	*p*
Prenatal depressive symptoms per sd															
Mother	1.18	1.08;1.29	<0.001	1.07	0.96;1.19	0.23	1.04	0.93;1.16	0.49	1.01	0.90;1.13	0.75	1.06	0.94;1.19	0.36
Father	1.15	1.05;1.26	0.002	1.07	0.96;1.19	0.22	1.05	0.94;1.17	0.33	1.01	0.92;1.13	0.74	1.04	0.93;1.16	0.52
Prenatal hostility symptoms per sd															
Mother				1.18	1.05;1.32	0.004	1.15	1.02;1.29	0.02	1.12	1.00;1.26	0.07	1.04	0.92;1.17	0.52
Father				1.15	1.04;1.28	0.008	1.14	1.02;1.27	0.02	1.13	1.01;1.26	0.03	1.06	0.95;1.19	0.31
Prenatal family functioning per sd															
Mother							1.28	1.15;1.42	<0.001	1.25	1.12;1.39	<0.001	1.23	1.10;1.37	<0.001
Father							0.99	0.89;1.11	0.92	0.97	0.87;1.08	0.65	0.96	0.83;1.05	0.51
Postnatal depressive symptoms per sd (3years after birth)															
Mother										1.24	1.13;1.36	<0.001	1.07	0.96;1.20	0.28
Father										1.11	1.01;1.22	0.02	0.99	0.88;1.10	0.82
Postnatal hostility symptoms per sd (3years after birth)															
Mother													1.35	1.20;1.52	<0.001
Father													1.30	1.17;1.46	<0.001

Reference group = children with Internalizing Problem scores below the 80th percentile on the Child Behavior Checklist.

[a] Model 1; prenatal depressive symptoms score of mother and father, Model 2; model 1 + prenatal symptoms of hostility score of mother and father, Model 3; model 2 + prenatal family functioning reported by mother and father, Model 4; model 3 + postnatal depressive symptoms score of mother and father, Model 5; model 4 + postnatal symptoms of hostility score of mother and father. All reported per standard deviation to facilitate the comparison of these measurements.

All analyses were adjusted for child gender, birth weight, birth order, ethnicity, child age at questionnaire, maternal smoking and alcohol use during pregnancy, parental age and parental educational level. Only mutually adjusted results are reported. Small differences in numbers due to the exclusion of outliers in postnatal psychological symptoms.

Abbreviations: OR; odds ratio, CI; confidence interval, p; p-value

[b]OR's represent the increased risk of internalizing problems per standard deviation (sd) increase of the determinants.

1.52, p-value <0.001), and postnatal symptoms of hostility of father (OR 1.30, 95%CI 1.17;1.46, *p* value <0.001) were all independently associated with an increased risk of child internalizing problems. The same pattern was found for the associations between the determinants and child externalizing problems (table 4); postnatal symptoms of hostility of the mother (OR 1.34, 95%CI 1.20; 1.50, *p* value <0.001) and of the father (OR 1.33, 95%CI 1.19; 1.48, *p* value <0.001) independently contributed to the likelihood of child externalizing problems. Also, the effects of the determinants on the risk of child internalizing problems and child externalizing problems were not significantly different, since the 84% confidence intervals of the ORs overlap (data not shown).

The analyses of parental psychopathology symptoms and prenatal family functioning with subtypes of internalizing problems and externalizing problems showed that postnatal symptoms of hostility of mothers and fathers each significantly increased the likelihood of all six subtypes of child emotional and behavioural problems at the age of three years (OR range 1.15-1.37) (see supplementary material Tables 2 and 3). Next to these significant observations, prenatal depressive symptoms of the mother were primarily related to anxious/depressed behaviour and emotionally reactive behaviour. Prenatal symptoms of hostility of the mother predicted child aggressive behaviour, whereas prenatal symptoms of hostility of the father were significantly associated with emotionally reactive behaviour of the child. Furthermore, prenatal family functioning reported by the mother was associated with anxious/depressed behaviour, emotionally reactive behaviour and somatic complaints, whereas prenatal family functioning reported by the father was related to somatic complaints of children (see supplementary material Tables 2 and 3).

DISCUSSION

This study examined the risk of prenatal and postnatal parental depressive symptoms, prenatal and postnatal parental hostility and prenatal family functioning for emotional and behavioural problems in young children. We first evaluate prenatal and postnatal effects of parental psychological symptoms. The associations of parental prenatal depressive symptoms and prenatal hostility of the parents with child internalizing and externalizing problems were not independent of the effect of postnatal parental hostility with the outcome. This may suggest that parent-child interaction is essential to determine the impact of parental psychopathology on children, or that it is easier to detect an immediate effect than a distant effect of a stressor on child development. However, these findings make it also more likely that the effects of postnatal parental symptoms of hostility on child behaviour are causal, because some form of hostility in the parent was already present before birth of the child. Child emotional and behavioural problems can be a source of postnatal parental psychological problems [12-13], but this reasoning

Table 4 The association of family functioning and parental psychopathology with child externalizing problems as reported by both parents in mutually adjusted successive models.

	Child Externalizing Problems at 3years reported by both parents (per sd)														
Successive	model 1 (n=2698)			model 2 (n=2698)			model 3 (n=2698)			model 4 (n=2687)			model 5[a] (n=2685)		
models	OR[b]	95% CI	*p*	OR[b]	95% CI	*p*	OR[b]	95% CI	*p*	OR[b]	95% CI	*p*	OR[b]	95% CI	*p*
Prenatal depressive symptoms per sd															
Mother	1.19	1.09;1.30	<0.001	1.09	0.98;.1.22	0.14	1.06	0.95;1.18	0.29	1.03	0.92;1.16	0.59	1.07	0.95;1.21	0.25
Father	1.12	1.02;1.23	0.009	1.04	0.94;1.16	0.48	1.03	0.96;1.15	0.60	1.01	0.91;1.13	0.88	1.03	0.92;1.15	0.63
Prenatal hostility symptoms per sd															
Mother				1.19	1.06;1.33	0.003	1.17	1.04;1.31	0.006	1.15	1.02;1.29	0.02	1.07	0.95;1.21	0.25
Father				1.16	1.04;1.29	0.005	1.15	1.03;1.28	0.013	1.12	1.00;1.25	0.04	1.05	0.94;1.18	0.37
Prenatal family functioning per sd															
Mother							1.15	1.04;1.28	0.010	1.11	1.02;1.29	0.05	1.10	0.99;1.23	0.08
Father							1.04	0.93;1.16	0.494	1.01	0.90;1.13	0.79	1.00	0.89;1.12	0.97
Postnatal depressive symptoms per sd (3years after birth)															
Mother										1.19	1.08;1.31	<0.001	1.02	0.91;1.14	0.68
Father										1.15	1.05;1.26	0.004	1.00	0.90;1.12	0.93
Postnatal hostility symptoms per sd (3years after birth)															
Mother													1.34	1.20;1.50	<0.001
Father													1.33	1.19;1.48	<0.001

Reference group = children with Externalizing Problem scores below the 80th percentile on the Child Behavior Checklist.

[a] Model 1; prenatal depressive symptoms score of mother and father, Model 2; model 1 + prenatal symptoms of hostility score of mother and father, Model 3; model 2 + prenatal family functioning reported by mother and father, Model 4; model 3 + postnatal depressive symptoms score of mother and father, Model 5; model 4 + postnatal symptoms of hostility score of mother and father. All reported per standard deviation to facilitate the comparison of these measurements.

All analyses were adjusted for child gender, birth weight, birth order, ethnicity, child age at questionnaire, maternal smoking and alcohol use during pregnancy, parental age and parental educational level. Only mutually adjusted results are reported. Small differences in numbers due to the exclusion of outliers in postnatal psychological symptoms.

Abbreviations: OR; odds ratio, CI; confidence interval, p; p-value

[b] OR's represent the increased risk of externalizing problems per standard deviation (sd) increase of the determinants.

cannot account for parental hostility prior to birth of the offspring, thus reverse causality is less likely.

Our second hypothesis posited that parental depressive symptoms, parental hostility and family functioning would have independent effects on child development. Although initially significant, the effects of parental depressive symptoms on child internalizing problems and child externalizing problems were accounted for by postnatal parental hostility. Hence, when analysed simultaneously with parental depressive symptoms, the impact of parental hostility after birth on child development seemed larger. However, we cannot conclude that depressive symptoms of the parents do not affect these families. It has been reported that persons with a depression show more nonverbal expressions of hostility in their interactions than non depressed persons [31]. In our study, this comorbidity was reflected by moderately strong correlations between depressive symptoms and symptoms of hostility. Also, in the subtype analyses, prenatal maternal depressive symptoms significantly predicted child anxious/depressed behaviour and emotionally reactive behaviour, independent of parental hostility.

We also report that poor family functioning during pregnancy experienced by the mother increases the risk of child internalizing problems, independently of the increased risk associated with parental postnatal hostility. The association between family functioning and child behavioural problems has been previously documented [15, 32]. In this field of research, many studies however focused on the combined effect of family functioning and parental psychopathology on child development [14, 16, 19-20, 32]. Our findings seem to underscore the importance of family life to child development, next to the effect of parental symptoms of psychopathology. Interestingly, this association seems specific for child internalizing problems, as we did not find an independent effect of prenatal family functioning on child externalizing problems.

To test the third hypothesis, we focused on the effects of maternal risk factors and the effects of paternal risk factors on child emotional and behavioural problems. Remarkably, the postnatal effect of father's hostility was similar to the effect estimate of mother's hostility on the likelihood of child internalizing and externalizing problems. These findings underscore the importance of father's behaviour on child development, and point to the fact that even subtle hostility by father affects their children. In contrast, father's experience of family functioning was not significantly associated with child internalizing problems. Fathers may have different views on family functioning compared to mothers. In our study, however, mothers and fathers report quite similar on family functioning in terms of total range of scores and mean scores. As both parents' reports of family functioning were included in one regression model (table 3), it may also be that the effect of father's experience of family functioning is captured in mother's experience of family functioning.

Two methodological considerations need to be discussed. First, we want to address the specificity of our findings. The associations of parental depressive symptoms and

hostility with child internalizing and externalizing problems were not specific; other BSI subscales also predicted child problems. This indicates strongly that these scales partly represent general psychopathology. We also found similar effects of postnatal parental hostility on child internalizing and child externalizing problems. If anything, family functioning reported by the mother was associated with a somewhat higher risk of internalizing problems compared with externalizing problems. This absence of effect specificity is not surprising, given the high level of comorbidity among psychiatric disorders in children [33]. Recently, Kessler and colleagues reported, even in adults, significant associations of "virtually all temporally primary lifetime disorders predicting subsequent onset of other disorders" in a study of lifetime comorbidity [34].

Secondly, the question of a multiple testing problem must be discussed. We studied the effect of several related risk factors on child emotional and behavioural problems. However, the tests do not constitute independent hypotheses. Hence we did not adjust for multiple testing. If we, however, would apply a Bonferroni correction, the corrected alpha for chance is 0.05/10= 0.005 which would not change the interpretation of our findings. The *p* values of the significant associations of postnatal parental hostility and child problems presented in Tables 3 and 4 are smaller than 0.001. Moreover, appendix Tables 2 and 3 present additional analyses of syndrome scales that further explore the results obtained with the broadband scale Internalizing and Externalizing. Hence, the aim here was not to test more hypotheses, but to explain the results observed.

The present study has several strengths. First, both mother and father participated in this study, and thus information about depressive symptoms, hostility and family functioning of both parents was available. This enabled us to study maternal and paternal effects on child development separately. Second, child emotional and behavioural problems were assessed separately by mother and father. Using multiple informants increased the reliability of our findings, and reduced the risk of reporter bias. Since assessment of behaviour will always be subjective, reporter bias may occur if, for instance, a depression of the mother influences her view on her child's behaviour [35]. Third, our study was embedded in a large birth cohort, which made it possible to adjust for numerous confounders. Besides these strengths, this study has also limitations. First, our response analyses showed that selection occurred toward well functioning families with a higher social economic status (SES). As partner participation is higher in families with higher SES, our study was prone to represent more well functioning families [36]. Second, observational measurements in this large cohort were not feasible. Therefore, we relied on report of mothers and fathers on psychological symptoms, family functioning and child behaviour. Yet, we used validated questionnaires with good reliability and validity. Third, as this study was performed in a fairly healthy population we should be careful generalizing our findings to clinical populations.

In conclusion, we found that family functioning experienced by the mother and postnatal hostile behaviour of parents independently contributed to the risk of internalizing

problems in 3-year-old children. Parental postnatal hostile behaviour was also related to child externalizing problems. Interestingly, parental hostility accounted for the effect of parental depressive symptoms on child internalizing and externalizing problems. These findings suggest that prevention and intervention strategies should focus on psychological symptoms of both mothers and fathers, in particular on hostile behaviour, in families with young children.

SUPPLEMENTARY MATERIAL

SM Table 1 Correlation* between parental depressive symptoms, parental symptoms of hostility and prenatal family functioning (n=2685).

	Prenatal depressive symptoms		*Prenatal hostility*		*Prenatal amily functioning*		*Postnatal depressive symptoms (3yrs.after birth)*		*Postnatal hostility (3yrs.after birth)*	
	mother	father	mother	father	mother	father	mother	father	mother	father
Prenatal depressive symptoms										
Mother	-	0.16	0.50	0.14	0.24	0.18	0.29	0.10	0.21	0.06
Father		-	0.16	0.44	0.17	0.25	0.13	0.27	0.09	0.16
Prenatal hostility										
Mother			-	0.16	0.24	0.17	0.21	0.10	0.29	0.09
Father				-	0.16	0.25	0.12	0.24	0.13	0.26
Prenatal family functioning										
Mother					-	0.39	0.19	0.15	0.17	0.11
Father						-	0.15	0.16	0.09	0.14
Postnatal depressive symptoms										
Mother							-	0.21	0.43	0.14
Father								-	0.11	0.44
Postnatal hostility										
Mother									-	0.21
Father										-

*Spearman's correlation coefficients, p-values all < 0.001

SM Table 2 The independent associations of parental psychopathology and prenatal family functioning with subtypes of child internalizing problems as reported by both parents (n=2685).

	Subtypes of Child Internalizing Problems at 3yrs. reported by both parents											
	anxious/depressed			somatic complaints			emotionally reactive			withdrawn		
	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*
Prenatal depressive symptoms (per sd)												
Mother	1.17	1.04;1.32	0.01	1.06	0.94;1.19	0.37	1.15	1.02;1.29	0.02	1.02	1.04;1.32	0.70
Father	1.02	0.91;1.14	0.78	1.09	0.98;1.22	0.11	0.975	0.87;1.09	0.66	0.97	0.94;1.19	0.66
Prenatal hostility(per sd)												
Mother	0.94	0.83;1,07	0.33	1.08	0.96;1.22	0.23	0.978	0.87;1.10	0.72	1.08	0.81;1.03	0.20
Father	1.08	0.96;1.21	0.20	1.06	0.95;1.19	0.35	1.18	1.06;1.32	0.003	1.02	0.96;1.21	0.72
Prenatal family functioning(per sd)												
Mother	1.17	1.05;1.31	0.005	1.18	1.06;1.32	0.004	1.16	1.04;1.29	0.007	1.09	1.06;1.32	0.11
Father	1.05	0.94;1.18	0.41	0.87	0.77;0.98	0.02	0.93	0.83;1.04	0.18	1.10	0.84;1.05	0.08
Postnatal depressive symptoms (per sd)(3yrs. after birth)												
Mother	1.00	0.89;1.12	0.98	1.04	0.93;1.17	0.51	1.03	0.92;1.15	0.60	1.19	1.10;1.38	0.002
Father	1.03	0.92;1.15	0.65	1.05	0.94;1.17	0.39	0.939	0.84;1.05	0.27	0.98	1.70;2.12	0.78
Postnatal hostility symptoms (per sd)(3yrs. after birth)												
Mother	1.19	1.06;1.34	0.004	1.22	1.09;1.37	0.001	1.32	1.18;1.48	<0.001	1.16	1.08;1.36	0.01
Father	1.20	1.07;1.34	0.002	1.13	1.01;1.26	0.03	1.33	1.19;1.48	<0.001	1.15	1.07;1.34	0.01

Reference group = children with syndrome scale scores below the 80th percentile on the Child Behavior Checklist. All analyses were adjusted for child gender, birth weight, birth order, ethnicity, child age at questionnaire, maternal smoking and alcohol use during pregnancy, parental age and parental educational level. Only mutually adjusted results are reported. Abbreviations: OR; odds ratio, CI; confidence interval, p; p-value

SM Table 3 The independent associations of parental psychopathology and prenatal family functioning with subtypes of child externalizing problems as reported by both parents (n=2685).

	Subtypes of Child Externalizing Problems at 3yrs. reported by both parents					
	attention problems			**aggressive behaviour**		
	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*
Prenatal depressive symptoms (per sd)						
Mother	1.07	0.95;1.21	0.293	1.05	0.93;1.18	0.430
Father	1.02	0.91;1.14	0.699	1.00	0.89;1.12	0.946
Prenatal hostility(per sd)						
Mother	0.95	0.84;1.08	0.401	1.15	1.02;1.29	0.021
Father	1.05	0.94;1.18	0.372	1.10	0.98;1.23	0.113
Prenatal family functioning(per sd)						
Mother	1.09	0.97;1.22	0.119	1.07	0.96;1.20	0.239
Father	0.946	0.84;1.06	0.359	1.00	0.90;1.12	0.848
Postnatal depressive symptoms (per sd)(3yrs. after birth)						
Mother	1.07	0.95;1.20	0.258	1.01	0.90;1.13	0.857
Father	0.91	0.81;1.03	0.121	0.99	0.89;1.11	0.857
Postnatal hostility symptoms (per sd)(3yrs. after birth)						
Mother	1.16	1.03;1.31	0.016	1.37	1.22;1.54	<0.001
Father	1.34	1.20;1.50	<0.001	1.26	1.13;1.40	<0.001

Reference group = children with syndrome scale scores below the 80th percentile on the Child Behavior Checklist. All analyses were adjusted for child gender, birth weight, birth order, ethnicity, child age at questionnaire, maternal smoking and alcohol use during pregnancy, parental age and parental educational level. Only mutually adjusted results are reported.Abbreviations: OR; odds ratio, CI; confidence interval, *p*; *p*-value. [a]OR's represent the increased risk of subscale scores per standard deviation (sd) increase of the determinants.

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Chapter 3

Genes

Chapter 3.1

FTO at rs9939609, food responsiveness, emotional control and symptoms of ADHD in preschool children.

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ABSTRACT

Background The *FTO* minor allele at rs9939609 has been associated with body mass index (BMI; weight (kg) /height $(m)^2$) in children from 5 years onwards, food intake, and eating behaviour. The high expression of *FTO* in the brain suggests that this gene may also be associated with behavioural phenotypes, such as impulsivity and self-control. We examined the effect of the *FTO* minor allele (A) at rs9939609 on eating behaviour, impulsivity and self-control in young children, thus before the BMI effect becomes apparent. **Methods**This study was embedded in the Generation R Study, a population-based cohort from fetal life onwards. 1718 children of European descent were genotyped for *FTO* at rs9939609. With logistic regression assuming an additive genetic model, we examined the association between the *FTO* minor allele and eating behaviour, impulsivity and self-control in preschool children. **Results** There was no relation between *FTO* at rs9939609 and child BMI at this age. The A allele at rs9939609 was associated with increased food responsiveness (OR 1.21, $p = 0.03$). Also, children with the A allele were less likely to have symptoms of ADHD (OR 0.74, $p = 0.01$) and showed more emotional self-control (OR 0.64, $p = 0.01$) compared to children without the A allele. **Conclusion** Our findings suggest that before the association between *FTO* and BMI becomes apparent, the *FTO* minor allele at rs9939609 leads to increased food responsiveness, a decreased risk for symptoms of ADHD and better emotional control. Future studies are needed to investigate whether these findings represent one single mechanism or reflect pleiotropic effects of *FTO*.

INTRODUCTION

Single nucleotide polymorphisms (SNPs) in intron 1 of the fat and obesity-associated transcript gene (*FTO*) in adults have consistently been associated with increased body mass index (BMI; weight (kg) /height $(m)^2$) and obesity [1,2]. In children the direction of the association between variance in *FTO* and BMI is age-dependent. In a large meta-analysis [3], the *FTO* rs9939609 minor allele was related to a lower body weight before the age of 2.5 years as compared to the body weight of carriers of the common allele. In this meta-analysis, the relation between the *FTO* minor allele and a higher BMI as observed in adults became only evident in children after the age of 5.5 years.

The biological mechanism responsible for the association between *FTO* and obesity remains to be determined. Animal studies showed that *Fto* genotypes in mice are associated with increased body weight, fat mass and food intake [4]. In children aged 4-11 years, variation in *FTO* at rs9939609 has been associated with increased food intake independent of body weight [5,6]. In 131 children aged 4-5 years, the *FTO* minor allele at rs9939609 (A) was associated with higher consumption of highly palatable food [7]. In a large sample of children aged 7-13 years, the AA genotype was associated with reduced satiety responsiveness [8]. Furthermore, children (6-19 years) with the *FTO* minor allele at rs9939609 were more likely to report loss of control over eating [9]. To the best of our knowledge, a possible effect of *FTO* on behavioural problems, such as impulsivity and other aspects of executive functioning, has not been studied yet.

FTO is highly conserved and widely expressed in both central and peripheral tissues [10]. The high expression of *FTO* in the hypothalamus, which is known to be involved in the control of energy homeostasis, possibly underlies the association between *FTO*, food intake and body weight [11]. *FTO* is also highly expressed in other brain areas such as the cortex, the hippocampus and the cerebellum. Hence, it has been suggested that *FTO* is involved in other functions as well [2,10]. This has been confirmed by reports showing an effect of *FTO* on overall-mortality, especially with an increased risk for diseases of the nervous system, which was independent of BMI [12]. Variation in *FTO* was also related to reduced brain volume in the healthy elderly [13].

In light of evidence for high expression of *FTO* in the brain and its relation with eating behaviour, the association of genetic variation in *FTO* with child behavioural phenotypes merits further investigation. Albeit inconsistently, child obesity has been associated with uncontrolled eating behaviour, and with emotional and behavioural problems such as impulsivity and attention-deficit/hyperactivity disorder (ADHD) [14,15]. On the other hand, Lawlor and colleagues reported an inverse association between BMI and psychological distress, in which common variance in *FTO* was used as instrumental variable [16]. Terracciano and colleagues reported an association between impulsivity and overweight, which was independent of *FTO* status. This suggests that *FTO* does not necessarily account for the association between impulsivity and overweight [17].

Our aim was to investigate the role of *FTO* at rs9939609 in eating behaviour, impulsivity and self-control in preschool children participating in a large population-based cohort. At this young age, no effect of common variance of *FTO* on BMI can be expected [3]. We hypothesized that the association between *FTO* at rs9939609 and eating behaviour may already be found in preschool children and possibly precedes the association between *FTO* and BMI. Given recent findings by Lawlor et al and Terracciano et al, *FTO* may not account for the association between BMI and behaviour, but instead may have an independent relation to behavioural phenotypes.

These hypotheses were tested in preschool children participating in a large population-based cohort. We explored the association between *FTO* at rs9939609, symptoms of ADHD and symptoms of Oppositional Defiant Disorder (ODD) at the age of 3 years. ADHD and ODD are both associated with impulsivity and the comorbidity of these disorders may largely be explained by shared genetic variance [18]. So we tested for the effect of the *FTO* minor allele on the risk of symptoms of ADHD and ODD to explore whether this effect was specific to ADHD or not. To study specific cognitive aspects of impulsive behaviour, we also examined the association between *FTO* at rs9939609 and components of executive functioning at the age of 4 years.

SUBJECTS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from foetal life onwards in Rotterdam, the Netherlands. The Generation R Study is a prospective population-based cohort study from fetal life onwards in the city of Rotterdam, the Netherlands. It was designed to identify early biological and environmental determinants of growth, development and health in fetal life and childhood. The Generation R Study has previously been described in detail [19,20] In short, all pregnant women living in the study area with a delivery date between April 2002 and January 2006 were eligible for enrollment in the Generation R Study. In total, 9,778 pregnant women were included, of whom 8,880 enrolled in the prenatal part of the study. The participating women gave birth to 9,745 live born children. Due to exclusion of participants in the pilot phase (12%) and because of withdrawal from the study (7%), 7,893 children participated in the postnatal phase of the Generation R Study. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam. Written informed consent was obtained from parents of the participating children.

Population of analysis

This study was restricted to children of Northern European descent, which was determined by principle component analyses of genome wide association data, as described

previously[19]. Principle component analyses yield factors that can be interpreted as the direction which maximizes the variance of the sample while being uncorrelated to previous components. Within the children of European descent (n=2650), *FTO* rs9939609 information was available in 2557 children. In 1718 (67%) of these children, information was available about child BMI, problem behaviour and eating behaviour. These 1718 children comprised the population of analysis.

Genotyping

DNA was collected from cord blood at birth. Participants were genotyped for *FTO* rs9939609. Genotyping was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg, Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C for 15 minutes, then 40 cycles of 94° C for 15 seconds and 60° C for 1 minute). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 97-99% of the samples. To confirm the accuracy of the genotyping 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1% for all genotypes. To check for potential contamination with maternal blood, sex was determined in male participants. Contamination occurred in < 1% of cases. Allele frequencies adhered to Hardy Weinberg Equilibrium (HWE) ($p = 0.405$).

Child eating behaviour

At the age of four years, eating behaviour was assessed using the Child Eating Behavior Questionnaire (CEBQ). The CEBQ [21] is designed to assess variation in eating style among children. In this 35-item instrument, parents rate their child's eating behaviour during the past month on a five-point Likert scale (1=never to 5=always). The CEBQ consists of four subscales that measure food approach behaviours: Emotional Overeating, Enjoyment of Food, Food Responsiveness, and Desire to Drink and three subscales that quantify food-avoidant responses: Emotional Undereating, Satiety Responsiveness, and Fussiness. Examples of items are "My child loves food" (Enjoyment of Food), "Even if my child is full, he finds room to eat his favourite food" (Food Responsiveness), and "My child has a big appetite" (Satiety Responsiveness). The CEBQ data has good psychometric properties, such as concurrent validity with actual eating behaviour, test-retest reliability, and stability over time [22] .

The Cronbach's alpha of all items was 0.99. Genetic variation in the *FTO* gene has previously been associated with increased food intake and satiety responsiveness. Therefore, the scales related to food approach behaviours (Emotional Overeating, Enjoyment of Food, Food Responsiveness) and one subscale about food avoidance (Satiety Responsiveness) were selected to examine the effect of FTO and food related behaviour in

our sample. The alpha's of these subscales were all 0.99.The CEBQ scales could not be normalized and were analyzed as dichotomized variables with a cut off at the highest 20 percent of the item scores in line with the CBCL cut-off.

Child behaviour

The Child Behaviour Checklist/1½-5 (CBCL/1½-5) was used to obtain standardized parent reports of children's emotional and behavioural problems at the age of 3 years. This questionnaire contains 99 items, which are scored on a three-point scale: 0 = not true, 1 = somewhat true or sometimes true, and 2 = very true or often true, based on the two preceding months. The CBCL/1½-5 comprises 5 DSM-oriented Scales; Affective Problems, Anxiety Problems, Pervasive Developmental Problems, Attention Deficit/ Hyperactivity Problems and Oppositional Defiant Problems. The psychometric properties of the CBCL are well established [23]. The Cronbach's alpha based on the 99 items was 0.92. In this study we used the DSM-oriented scales Attention Deficit/ Hyperactivity Problems (ADHD) and Oppositional Defiant Problems (ODD), which comprise problems with impulsivity and self-control. The alpha's of ADHD was 0.75 and 0.64 for ODD. These scales could not be normalized and were analyzed as dichotomous variables. In the absence of Dutch norm scores, we defined a non-optimal score as the highest 20 percent of the item scores in line with previous studies [24].

Child executive functioning

The Behavior Rating Inventory of Executive Function-Preschool Version (BRIEF-P) was used to assess child executive function at 4 years. The BRIEF-P is a questionnaire for parents to assess the executive function behaviour in a broad age range of preschoolers (2-5 years) [25,26]. It contains 63 items within five related clinical scales that measure different aspects of executive functioning: emotional control, inhibit, shift, working memory, and plan/organize. The parents were asked to rate problematic behaviour of the child in the preceding month on a three-point scale (never, sometimes, and often). In the present study, we used the clinical scales emotional control, inhibit and shift as in general young children show most problems on these components of ADHD. The Cronbach's alpha of all 63 items was 0.94. The alpha's of the clinical scales were; "inhibit" 0.88, "shift" 0.81, "emotional control" 0.82. Based on sex and age, the raw scores of the clinical scales are transformed into T scores. A T score of 65 represents 1.5 standard deviations above the mean, and distinguishes non-clinical scores from clinical scores.

Covariates

Gestational age was established by fetal ultrasound examinations. Information about birth weight and gender was obtained from midwife and hospital registries at birth; information about maternal age, marital status, parity and educational level was ob-

tained by questionnaire. The highest completed education (primary school, secondary school or higher education) determined the educational level. At the age of three and four years, trained staff in the community health centers obtained children's weight and length using standardized procedures. Child body mass index (BMI; kg/m^2) was calculated and sex and age adjusted standard deviation scores (SDS) of the Dutch reference growth curves were obtained (Growth Analyser 3.0, Dutch Growth Research Foundation). These covariates were not selected to adjust for confounding, but to describe the study sample.

Statistical analysis

We examined selective non-response by comparing characteristics between mothers and children included (n=1718) and those excluded (n=839) from this study using chi-square statistics for categorical variables, independent t-tests for normally distributed continuous variables and Mann Whitney U-tests for non-normally distributed continuous variables. Using the same tests, we compared characteristics of mothers and children in this study by *FTO* genotype. The correlation between the outcome variables (symptoms of ADHD and ODD, executive function and eating behaviour), was calculated with the Spearman's correlation coefficient for non-parametric variables (two-tailed). Logistic regression analyses were performed to test the association between *FTO* rs9939609 and child eating behaviour. The analyses were run under the assumption of an additive genetic model, which also optimizes the power. In addition, we also present results for a three-categorical genetic model with the TT genotype as reference category to explore recessive or dominant effects. Logistic regression was also used to test for an association between *FTO* at rs9939609 and symptoms of ADHD and ODD, and emotional control, inhibition and shift. The level of significance for all analyses was set at $\alpha = 0.05$. All statistical analyses were carried out using PASW Statistics, version 17.0 for Windows [27].

Non-response analysis

Children excluded from our study (n=839) had on average younger (30.5 vs. 32.1 years, $t = 9.23$, $p < 0.001$) and less highly educated mothers (28.5 vs. 43.0%, $\chi 2 = 52.43$(1df), $p < 0.001$), and had a lower birth weight (3503 versus 3573 g, t=3.30, $p = 0.001$) than children included in our study (n=1718). The distribution of sex, birth order, BMI and the minor allele frequency of rs9939609 (61.4 vs. 60.6%, $\chi 2 = 0.147$(1df), $p = 0.70$) did not differ amongst these two groups.

RESULTS

The mother and child characteristics are presented in table 1. The distribution of the characteristics did not differ between children homozygous for the T allele (TT), hetero-

zygous children (AT) or children homozygous for the A allele (AA) (table 1). Child BMI did not differ according to the *FTO* genotype at rs9939609.

Table 2 presents the correlation between the outcome measurements. It shows that symptoms of ADHD and ODD were positively correlated with emotional control and inhibition. Symptoms of ADHD and ODD were not significantly correlated with food approach, and symptoms of ADHD weakly correlated with satiety responsiveness (food avoidance) (Spearman's rho 0.06, *p* value <0.01). Child BMI was positively correlated to food responsiveness and enjoyment of food. There was a negative correlation between child BMI and satiety responsiveness. Child BMI was not significantly correlated to the other behavioural phenotypes.

Table 1 Sample characteristics by *FTO* rs9939609 genotype (n=1718).

	Child *FTO* rs9939609			
	TT (n=677)	**AT (n=790)**	**AA (n=251)**	***p* value**
Mother [a,c]				
Age at child birth (years)	32.1(3.8)	32.2(4.1)	32.1(3.3)	0.99
Education				
higher education (%)	42.3	44.5	41.1	0.52
Marital status				
married/living together %	96.2	96.2	97.9	0.42
Child [a,c]				
Gestational age (weeks) [b]	40.4(32.7;43.0)	40.4(29.9;43.4)	40.3(35.4;43.0)	0.67
Birth weight (gram)	3575(506)	3566(495)	3589(530)	0.82
Birth order				
first child (%)	60.6	59.7	61.0	0.91
Gender				
boys (%)	48.4	51.8	55.0	0.17
Child age				
3 years assessment	36.47(0.04)	36.48 (0.04)	36.48 (0.07)	0.98
4 years assessment	468.42 (0.03)	48.53 (0.04)	48.39 (0.05)	0.05
BMI sd_score				
BMI age 3	0.25(0.99)	0.21(0.92)	0.24(0.98)	0.68
BMI age 4	0.12(0.90)	0.08(0.91)	0.16(0.88)	0.51
Overweight or obesity (%)[d]	10.3	11.1	12.4	0.68

[a]mean (standard deviation) unless otherwise indicated
[b]median (100% range)
[c]with the chi-square statistic for categorical variables, one-way ANOVA for normally distributed continuous variables and the Kruskal-Wallis test for non-normally distributed
continuous variables.
[d] overweight or obesity is defined as a BMI-sds >1.10.

Table 2 Correlation between child BMI, child emotional and behavioural problems, executive function and eating behaviour.

	BMI	Symptoms of ADHD	Symptoms of ODD	Emotional control	Inhibition	Shift	Food response	Enjoyment of food	Emotional overeating	Satiety responsiveness
	n=1718	n=1717	n=1714	n=1638	n=1621	n=1636	n=1718	n=1718	n=1718	n=1718
BMI	-	-0.01	0.03	-0.02	0.02	-0.02	0.23***	0.06**	0.02	-0.02***
Symptoms of ADHD		-	0.35**	0.21**	0.31**	0.04	0.04	0.01	0.04	0.06*
Symptoms of ODD			-	0.27**	0.23**	0.08**	0.02	-0.03	0.01	0.01
Emotional control				-	0.31**	0.30**	0.05	-0.04	0.05	0.03
Inhibition					-	0.13**	0.03	0.01	0.06	0.06*
Shift						-	0.003	0.01	0.03	0.002
Food responsiveness							-	0.32	0.33**	0.09**
Enjoyment of food								-	0.29**	0.19**
Emotional overeating									-	0.27**
Satiety responsiveness										-

Spearman correlation coefficient (two tailed) * <0.05, ** <0.01 *** <0.001

Table 3 presents the association between the *FTO* minor allele at rs9939609 and child eating behaviour at 4 years. The minor allele was associated with food responsiveness (OR 1.21, 95%CI 1.02;1.43, *p* value = 0.026), which did not materially change after adjustment for symptoms of ADHD (OR 1.22, 95%CI 1.04;1.45, *p* value = 0.018). This effect was slightly attenuated after adjustment for gender and child age (OR 1.17, 95%CI 0.98;1.40, *p* value = 0.09). However, child age and gender were not significantly associated with food responsiveness. Children with 2 copies of the minor allele (AA) were more likely to score high on food responsiveness (OR 1.45, 95%CI 1.03;2.05, *p* value = 0.04) compared to children without the minor allele. Children with the minor allele did not have an increased likelihood for high scores on enjoyment of food (OR 1.08, 95%CI 0.88;1.34, *p* value = 0.46) or emotional eating (OR 1.10, 95% CI 0.96;1.40, *p* value = 0.13). Neither was the minor allele related to satiety responsiveness (OR 1.05, 95%CI 0.89;1.24, *p* value = 0.56). Child BMI was higher in children with high scores on food responsiveness (24% versus 7.6%, $\chi 2$ =77.8(1df), $p< 0.001$) and children with high scores on enjoyment of food (16.4% versus 10.6%, $\chi 2$ =6.8(1df), $p= 0.009$) compared to children with low scores on these phenotypes. The difference in BMI between children with high scores on satiety responsiveness compared to children with low scores was close to significant (8.3% versus 11.6%, $\chi 2$ =2.8(1df), $p= 0.09$ (see Supplementary Material (SM) Table 1).

In table 4, we present the association between the *FTO* minor allele at rs9939609 and symptoms of ADHD and ODD. The minor allele was associated with a lower risk for

symptoms of ADHD (OR 0.74, 95%CI 0.59; 0.93, *p* value = 0.009), which was not attenuated by adjustment for child age and gender (OR 0.74, 95%CI 0.59; 0.93, *p* value = 0.008). Preschool children with 2 *FTO* minor alleles (AA) were less likely to show symptoms of ADHD (OR 0.53, *p* value = 0.02) compared with children without the minor allele. The minor allele was not significantly associated with symptoms of ODD (OR 1.08, 95%CI 0.88; 1.34, *p* value = 0.46). Child BMI did not significantly differ between children with high scores and low scores on ADHD an ODD symptoms (see SM table 1).

Table 5 presents the association between the *FTO* minor allele at rs9939609 and executive function. The minor allele was associated with less problems with emotional control (OR 0.64, 95%CI 0.47;0.88, *p* value = 0.006), which was not attenuated by adjustment for symptoms of ADHD (OR 0.69, 95%CI 0.50;0.96, *p* value = 0.026) or child age and gender (OR 0.61, 95%CI 0.44;0.84, *p* value = 0.002). Children with the AA genotype were significantly less likely to have problems with emotional self-control (OR 0.31, 95%CI 0.13;0.74, *p* value = 0.01) compared to children without the minor allele. The association between the *FTO* minor allele, and problems with Inhibit and Shift did not reach significance. Child BMI did not significantly differ between children with high scores and low scores on these components of executive functioning (see SM table 1).

Table 3 Associations of child *FTO* at rs9939609 with child eating behaviour at 4 years (n=1718).

	Food responsiveness			**Enjoyment of Food**			**Emotional overeating**			**Satiety responsiveness**		
***FTO* at rs9939609**	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*
additive model	1.21	1.02;1.43	0.026	1.08	0.88;1.34	0.46	1.16	0.96;1.40	0.13	1.05	0.89;1.25	0.56
per genotype												
TT	ref			ref			ref			ref		
AT	1.23	0.95;1.59	0.12	1.22	0.88;1.68	0.23	1.17	0.87;1.56	0.29	0.97	0.75;1.27	0.84
AA	1.45	1.03;2.05	0.04	1.10	0.70;1.74	0.69	1.34	0.90;1.98	0.15	1.16	0.81;1.66	0.42

Abbreviations: OR, odds ratio; 95%CI, 95% confidence interval; ref, reference.
[a]The ORs represent the increased risk of high scores on CEBQ subscales per *FTO* minor allele at rs9939609.

Table 4 Associations of child *FTO* at rs9939609 with behavioural problems at 3 years (n=1718).

	Symptoms of ADHD			**Symptoms of ODD**		
***FTO* at rs9939609**	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*
additive model	0.74	0.59;0.93	0.009	1.08	0.88;1.34	0.46
per genotype						
TT	ref			ref		
AT	0.77	0.56;1.05	0.10	1.53	1.11;2.12	0.01
AA	0.53	0.32;0.89	0.02	0.93	0.57;1.54	0.78

Abbreviations: ADHD, attention deficit hyperactivity disorder; ODD, oppositional defiant disorder; OR, odds ratio; 95%CI, 95% confidence interval; ref, reference.
[a]The ORs represent the increased risk of high scores on CBCL DSM-oriented scales per *FTO* minor allele at rs9939609.

Table 5 Associations of child *FTO* at rs9939609 with child executive function at 4 years.

	problems with Emotional control (n=1637)			problems with Inhibition (n=1620)			problems with Shift (n=1636)		
***FTO* at rs9939609**	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*
additive model	0.64	0.47;0.88	0.006	0.81	0.57;1.16	0.26	0.79	0.58;1.09	0.15
per genotype	OR[a]	95%CI	*p*	OR[a]	95%CI	*p*	OR[a]	95%CI	*P*
TT	ref			ref			ref		
AT	0.75	0.50;1.15	0.18	0.73	0.43;1.22	0.22	0.84	0.54;1.32	0.45
AA	0.31	0.13;0.74	0.01	0.73	0.34;1.55	0.41	0.59	0.28;1.22	0.15

Abbreviations: OR, odds ratio; 95%CI, 95% confidence interval; ref, reference.
[a] The ORs represent the increased risk of clinical scores on BRIEF-P Emotional Control and Inhibition per *FTO* minor allele at rs9939609.

DISCUSSION

In a large population-based cohort, children with 2 copies of the *FTO* minor allele (A) at rs9939609 were less likely to have symptoms of ADHD and had less problems with emotional self-control compared with the other children. Independent of symptoms of ADHD, the *FTO* minor allele was associated with increased food responsiveness.

First, we explored the association between *FTO*, preschool BMI and eating behaviour. As expected at this young age, there was no significant association between the *FTO* minor allele at rs9939609 and child BMI. Children of preschool age with high scores on food responsiveness and enjoyment of food already had a higher BMI compared to low scoring children. Even in these young children, the *FTO* minor allele was already associated with increased food responsiveness. The direction of the effect of the minor allele on eating behaviour in our study is similar to previous reports about *FTO* and behaviour related to food approach. As designed by Wardle and colleagues, the scale "food responsiveness" detects levels of maladaptive appetite and assesses the tendency to eat when prompted by external cues [22]. It seems plausible that this type of eating behaviour accounts at least partially for the relation of *FTO* with increased food intake. The association of the minor allele at rs9939609 with food responsiveness was not altered by adjustment for child symptoms of ADHD, which indicates that the effect of this minor allele on eating behaviour was not explained by the association between *FTO* and symptoms of ADHD.

Second, we tested for an effect of the *FTO* minor allele at rs9939609 on symptoms of ADHD and ODD. Independent of eating behaviour, the *FTO* minor allele was associated with a decreased likelihood of symptoms of ADHD. Possibly, this effect is specific to certain phenotypes of ADHD, as no association with ODD was found, which is often

co-morbid to some forms of ADHD. Most plausibly, the association between the AT genotype and ODD is false-positive, since we had no hypothesis on prior data to suggest that heterozygosity at rs9939609 is associated with negative or positive outcomes. Recently, two studies used common variance in *FTO* as instrumental variable in mendelian randomization designs to examine the association between BMI and psychopathology in adults. In a cohort of London-based civil servants, variance in *FTO* was associated with long-term obesity and independently with common mental disorders [28]. However, this association was found in men only and with a different variant of *FTO* (rs1421085) than investigated in the current study. In a study including more than 50,000 subjects, the mendelian randomization design showed an inverse association between common variance in *FTO*, BMI, and psychological distress [16]. In line with this inverse association reported by Lawlor and colleagues, we reported an inverse association between *FTO* at rs9939609 and symptoms of ADHD. Terraccino and colleagues reported that in persons 14 to 94 years of age impulsivity was associated with higher BMI, independent of *FTO* status. An association between *FTO* and impulsivity was, however, not tested. In our study, child BMI did not differ according to low or high scores on symptoms of ADHD. This may be partly explained by parental control over eating in these young children, which are on average also fairly active. It seems less likely that the use of stimulant medication influenced this association, since Dutch children at this age are not yet diagnosed with ADHD and unlikely to receive stimulant medication.

In young children, ADHD is characterized by temperamental problems in self-regulation, shown by inattentiveness, overactivity and impulsiveness [29]. Hence, to test for the effect of FTO on specific cognitive aspects of ADHD, we examined the association between the minor allele of FTO, and emotional control, inhibition and shift at the age of 4 years. We were able to show that the minor allele of FTO at rs9939609 is associated with better emotional control, independent of symptoms of ADHD. The work of Sovio and colleagues may help explain these findings. They found a relation between the minor allele at rs9939609 and the age of the adiposity rebound in young children [3]. The adiposity rebound marks the transition from a decline in BMI to a rise in BMI, which on average occurs around the age of 5 to 6 years. An earlier adiposity rebound has been associated with obesity and accelerated growth [30]. Hence, it was posited that carriers of this minor allele might be more developmentally advanced than wild type carriers [3]. Our findings may support this theory, since children differed in their ability to control their behaviour according to the minor allele of *FTO* at rs9939609. A child's ability to control its behaviour is expected to improve with age and thus reflects a stage of development.

Alternatively, our findings may be explained by specific behaviour of children with the minor allele of *FTO*. Compared to non-carriers, these children tend to respond more to highly palatable food, which is known to stimulate dopamine pathways [31]. These dopamine pathways are thought to be involved in the pathophysiology of ADHD [32].

Hence, the eating behaviour of children with the *FTO* minor allele may reduce symptoms of ADHD by acting as a natural reward. Therefore, the relation between the *FTO* minor allele at rs9939609 and less symptoms of ADHD might actually be the result of specific eating habits, which one can view as "self-medication by eating". Finally, our findings may reflect pleiotrophy of *FTO*. Pleiotrophy refers to the effect of one genetic region on more than one phenotype, which is the result of a single gene that can be transcribed differently or a single gene product that can affect multiple phenotypes [33]. Clearly, further imaging and biomedical research is warranted to reveal the mechanism underlying these effects of *FTO*.

This study has considerable strengths, such as the large sample size and the minimization of possible bias of population heterogeneity by using GWAS data to select children of Northern European descent. Despite these strengths, several limitations should be discussed. First, the restriction to children of Northern European descent not only minimizes the bias by population heterogeneity, it also limits the generalizability of our findings to other populations. Second, observational measurements in this large cohort were not feasible. Therefore, we relied on maternal report of child behaviour, executive function and eating behaviour. Yet, we used validated questionnaires with good reliability and validity. Third, symptoms of ADHD and ODD were studied rather than clinical diagnoses based on the DSM IV classification. Future studies are needed to confirm our results in clinical populations. Fourth, the level of children's physical activity may influence the association between the FTO minor allele and BMI. Ruiz and colleagues showed that the FTO minor allele was associated with a higher BMI in less active adolescents only, and not in active adolescents [34]. Possibly, the association between the FTO minor allele and child behaviour is also influenced by physical activity. However, in our sample this information was not available at the age of 3 or 4 year, and thus this potential interaction could not be tested.

Overall, this study provides initial support for an association of the *FTO* minor allele at rs9939609 with decreased risk for symptoms of ADHD and less problems with emotional control in preschool children. Independent of symptoms of ADHD, *FTO* was also related to food responsiveness. Future research is needed to determine whether these findings can be explained by a single underlying mechanism such as accelerated development or self-medication by eating, or that they reflect the pleiotrophy of the *FTO* genotype.

SUPPLEMENTARY MATERIAL

SM Table 1 Percentage of overweight or obesity in groups of children with low scores versus high scores on behavioural phenotypes.

	Prevalence of Overweight or obesity[b]		Chi- square	*p* value
	Frequency of low behavioural symptoms score[a] %	Frequency of high behavioural symptoms score[a,] %		
Behavioural problem score				
Symptoms of ADHD	11.2	9.8	0.33	0.57
Symptoms of ODD	10.7	13.4	1.34	0.25
Executive functioning				
Emotional control	11.1	8.0	0.90	0.34
Inhibition	10.8	12.9	0.30	0.58
Shift	11.1	7.8	0.95	0.33
Eating behaviour				
Food responsiveness	7.6	24.0	77.8	<0.001
Enjoyment of food	10.6	16.4	6.82	0.009
Emotional overeating	11.0	11.1	0.004	0.95
Satiety responsiveness	11.6	8.3	2.97	0.09

[a] low symptoms scores are defined by scores < 80th percentile, high symptoms scores are defined by scores ≥ 80th percentile.
[b] overweight or obesity is defined by a BMI sd score > 1.10

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Chapter 3.2

Genetics of cortisol secretion and depressive symptoms: A candidate gene and genome wide association approach.

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ABSTRACT

Background Depressive patients often have altered cortisol secretion, but few studies have investigated genetic variants in relation to both cortisol secretion and depression. To identify genes related to both these conditions, we (1) tested the association of single nucleotide polymorphisms (SNPs) in hypothalamic-pituitary-adrenal-axis (HPA-axis) candidate genes with a summary measure of total cortisol secretion during the day ($cortisol_{AUC}$) (2) performed a genome wide association study (GWAS) of $cortisol_{AUC}$; and (3) tested the association of identified cortisol-related SNPs with depressive symptoms. **Methods** We analyzed data on candidate SNPs for the HPA-axis, genome-wide scans, cortisol secretion (n=1711) and depressive symptoms (the Centre for Epidemiology Studies Depression Scale, CES-D) (n=2928) in elderly persons of the Rotterdam Study. We used data from the Whitehall II study (n=2836) to replicate the GWAS findings. **Results** Of the 1456 SNPs in 33 candidate genes, minor alleles of 4 SNPs (rs9470080, rs9394309, rs7748266 and rs1360780) in the *FKBP5* gene were associated with a decreased $cortisol_{AUC}$ ($p<1 \times 10(-4)$ after correction for multiple testing using permutations). These SNPs were also associated with an increased risk of depressive symptoms (rs9470080: OR 1.19 (95%CI 1.0; 1.4). The GWAS for cortisol yielded 2 SNPs with p-values of 1x10(-06) (rs8062512, rs2252459), but these associations could not be replicated. **Conclusions** These results suggest that variation in the *FKBP5* gene is associated with both $cortisol_{AUC}$ and the likelihood of depressive symptoms.

INTRODUCTION

The diurnal secretion of cortisol is characterized by high levels in the morning followed by a decline in cortisol levels toward the evening, and reflects the total cortisol exposure during day time. After its release from the adrenal glands, cortisol exerts diverse actions including on the immune system, glucose metabolism and the central nervous system. The effect of cortisol on target organs is mediated by two types of receptor: the mineralocorticoid receptor (MR (NR3C12)) and the glucocorticoid receptor (GR (NR3C1)). In the brain, MR mediates the onset of the stress response, whereas GR is involved in the termination of the stress response. The heritability of diurnal cortisol secretion has been estimated at 62% [1].

Several studies have shown that depression is associated with hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis resulting in high cortisol levels [2-4] and abnormal cortisol secretion in response to standard stimuli, such as the DEX/CRH suppression test [5]. It has been suggested that these alterations in cortisol secretion are causally related to the development of depression and for this reason the identification of genes related to cortisol secretion may enhance the discovery of genes associated with depression [6]. Recent studies provide some support for this hypothesis. For example, single nucleotide polymorphisms (SNPs) in the MR gene have been associated with morning cortisol levels [7] and, in elderly subjects, with the prevalence of depressive symptoms [8]. SNPs within the GR gene have been associated with hypersensitivity to cortisol, the occurrence of depression, and the response to antidepressant treatment [9]. The FK506 binding protein 5 (FKBP5), which acts as co-chaperone of GR, is associated with the sensitivity of GR to cortisol and SNPs within the *FKBP5* gene have been shown to be associated with depression[10-11], treatment response and recurrence of depressive episodes [12]. However, most of these studies examined cortisol secretion under stressful circumstances without an assessment of basal cortisol secretion. In the current study, we sought to find genes related to cortisol secretion and ultimately to depressive symptoms. We measured saliva cortisol concentrations during the day, which approximate free plasma cortisol [13-14]. To prevent multiple testing and to increase precision, we applied a commonly used summary measure of total cortisol secretion during the day expressed as the area under the curve ($cortisol_{AUC}$) [15]. First, we performed a candidate gene study of $cortisol_{AUC}$. Candidate gene studies rely on biological knowledge and are able to examine the postulated association with more power as multiple testing is not so stringent as less tests are peformed due to prior selection. However, this approach does not identify new genes. Therefore, we also performed a hypothesis free GWAS to try to discover genes not previously associated with HPA-axis functioning. Third, we examined the effect of the SNPs associated with $cortisol_{AUC}$ in the candidate gene study and/or GWAS on the risk of depressive symptoms.

METHODS AND MATERIALS

Setting and study population

This study is embedded in the Rotterdam Study, an ongoing population-based cohort on risk factors for chronic diseases in the elderly. Detailed information on design, objectives and methods has been presented elsewhere [16]. The Medical Ethics Committee of the Erasmus Medical Center approved the Rotterdam study and written informed consent was obtained from all participants. Of the 3550 participants of the fourth study survey (2002-2004), genetic and cortisol data were available in 1711 persons. In 2928 participants, genetic data and information about depressive symptoms were available.

Candidate genes and genome-wide data

Based on the literature, we selected candidate genes known to be involved in central regulation of the HPA-axis, cortisol biosynthesis in the adrenal, or the clearance of cortisol from the circulation (table 2). To select SNPs within the candidate gene regions, we included 100kb at 5'and 3' of the genes to e.g. include regulatory regions. We then extracted these SNPs from the GWAS genotyping, using PLI NK v1.02 [17].

The genome-wide genotyping was done with the Illumina 550K array (Illumina, San Diego, CA, USA) in self-reported Caucasian individuals (sample call rate ≥97·5%) [18]. We excluded individuals for excess autosomal heterozygosity, mismatch between genotypic and phenotypic gender, and outliers identified by the identity-by-state (IBS) clustering analysis. SNPs were excluded when the minor allele frequency (MAF) was 1% or less, the Hardy-Weinberg equilibrium (HWE) p-value was smaller than 1 x 10 (-5), or the SNP call rate was 90% or less. This resulted in 530,683 SNPs.

Cortisol collection

Participants were asked to collect saliva samples at home using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). As described previously [19], participants received detailed oral and written instructions concerning the saliva sampling, and were asked to collect four saliva samples during one single weekday: at awakening, 30 minutes later, at 5 pm and at bedtime. Samples were stored in the freezer at minus 80^{o} C and later sent to the laboratory of Biopsychology, TU Dresden, Germany. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 6% and 9%, respectively. In line with a previous study [19], cortisol values above the 98th percentile in the original cortisol dataset were excluded.

Depressive symptoms

Depressive symptoms were assessed using the Centre for Epidemiology Studies Depression Scale (CES-D) which is a validated and reliable self-report measure [20]. The

CES-D consists of 20 items with total scores ranging from 0 to 60. We used a cut-off of 16 or higher to dichotomize depressive symptoms. This cut-off is indicative of clinically relevant symptoms and a sensitive threshold for depressive disorder [21].

Other covariates

Information about gender, age , educational level, smoking habits was assessed by interview, coronary heart disease (fatal or nonfatal myocardial infaction, a percutaneous transluminal coronary angioplasty, a coronary artery bypass graft, other forms of acute or chronic ischemic heart disease, sudden cardiac death, and death due to ventricular fibrillation and congestive heart failure), diabetes mellitus type II was assessed by fasting glucose levels and the use of antidiabetic medication, use of hypnotics, antidepressants and antipsychotics was assessed by by cabinet check.

GWAS Replication Study

The Whitehall II Cohort recruited participants between 1985 and 1988 (phase 1) from 20 London-based civil service departments. Data reported here are from the phase 7 (2002-2004) data collection when large-scale genotyping was undertaken for the first time. Saliva samples were returned by 90.1% (n=4609) of the participants who attended the screening clinic [22]. Details of the clinical assessment and cohort profile have been reported elsewhere [23].

The protocol of saliva collection has been described previously [22]. Briefly, participants were requested to provide six saliva samples over the course of a normal weekday at awakening, waking +30 min, waking + 2.5 h, waking + 8 h, waking + 12 h and bedtime. Salivary cortisol levels were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany).

SNPs were genotyped at kbioscience (http://www.kbioscience.co.uk) using KASPar chemistry, which is a competitive allele-specific PCR SNP genotyping system using FRET quencher cassette oligos. Blind duplicates, plate-identifying blank wells and Hardy-Weinberg equilibrium tests were used as quality control tests. Cortisol data and genotype data were available in 2836 white participants of the Whitehall II Study.

Statistical analyses

First, we determined the total diurnal cortisol secretion with respect to the ground expressed by the area under the curve ($cortisol_{AUC}$). This is given by the cortisol measurements in nmol/L on the y-axis and the time between the cortisol measurements on the x-axis [15]. To correct for differences in length of total sampling interval, the $cortisol_{AUC}$ was divided by time awake during the day in hours. The unit of $cortisol_{AUC}$ is thus (nmol*hr/L)/hr = nmol/L.

Second, we performed a linear regression analysis to examine the association of SNPs of candidate genes with the $cortisol_{AUC}$. P-values of these associations were corrected for

multiple testing using the Max(T) permutation procedure (10,000 permutations). The regression analysis and the permutation procedure were performed using PLINK v1.02 [17].

Third, we performed a genome-wide quantitative trait analysis of diurnal cortisol secretion using PLI NK v1.02 [17]. The Hardy–Weinberg equilibrium (HWE) p-value was set at 1 x 10(-4), the maximum percentage SNPs per person missing at 2%, and the threshold for the minor allele frequency (MAF) at 1%. We also performed a GWAS in females and males separately.

Fourth, to replicate our GWAS findings we selected SNPs of the GWAS based on p-values (< 1 x 10 (-5) and LD between SNPs. We also selected SNPs of the GWAS that were located in candidate genes and showed a strong association (p-value <1 x 10(-4)) with the cortisol$_{AUC}$. We tested the association of these selected SNPs of the GWAS with cortisol data of the Whitehall II Study using linear regression analyses in an additive model (SPSS Inc, Chicago, IL, USA). Next, we performed a meta-analysis using the inverse Z score method assuming fixed effects (R 2.8.1).

Fifth, we tested the association of statistically significantly associated SNPs in the candidate gene study or the GWAS with clinically relevant depressive symptoms in participants of the Rotterdam Study using logistic regression analysis in PLINK v1.02 [17], adjusted for age and gender. To examine if the association of SNPs and depressive symptoms could be explained by other characteristics, we tested if non-carriers and carriers differed with respect to gender, age, educational level, smoking, diabetes mellitus type II, coronary heart disease, or the use of hypnotics, antidepressants and antipsychotics.

RESULTS

GWAS sample and replication sample

Table 1 presents the characteristics of participants of the GWAS discovery sample and the replication sample. Participants of the GWAS discovery sample were more likely female, and were, on average, older than participants in the replication sample. Furthermore, people in the GWAS discovery sample were more likely to smoke, less highly educated, and were more likely to report diabetes mellitus type II, coronary heart disease and the use of hypnotics. Also, there were small differences in cortisol levels between the two samples.

The candidate gene study

The list of 33 candidate genes, the number of SNPs within these genes (1456 in total), and their position are presented in table 2. Initially, 96 of 1456 SNPs were associated with the cortisol$_{AUC}$ with a p-value less than or equal to 0.05 (see supplementary material (SM) table 2 available online), of which table 3 shows the top 10 SNPs located in 7 genes;

the FK506 binding protein 5 (*FKBP5*) gene, the melanocortin 4 receptor (*MC4R*) gene, the corticotrophin-releasing hormone receptor 2 (*CRHR2*) gene, the arginine vasopressin (*AVP*) gene, the steroid-5-alpha-reductase (*SRD5A1*) gene, the mineralocorticoid receptor (*NR3C2*) gene, and the 3-beta hydroxysteroid dehydrogenase II (*HSD3B2*) gene. After adjustment for multiple testing, 4 SNPs (rs9470080, rs9394309, rs7748266 and rs1360780) showed significantly lower $cortisol_{AUC}$ per minor allele. These SNPs are all located in the *FKBP5* gene and in strong LD with each other.

GWAS of cortisol and the replication study

Table 4 presents the results of the GWAS, the replication and the meta-analysis. The most significant association was found between rs8026512 on chromosome 15 with a lower $cortisol_{AUC}$ per C allele (beta=-0.57, p-value 6.04 x 10(-6)). The second hit (rs11630255) was in LD with rs8026512 and showed a similar association with cortisol (beta=-0.57, p-value 7.45 x 10(-6)) so was not selected for replication. The third hit with a p-value < 1 x 10(-5) (rs2252459) was associated with a higher $cortisol_{AUC}$ per C allele (beta 0.53, p-value 8.75 x 1(-6)). It is located in intron 3 of the activated leukocyte cell adhesion molecule (*ALCAM*) gene on chromosome 3 (3q13.1). There were no other hits with a p-value <1 x 10(-5). Within the first ten hits of the GWAS, we found two SNPs (rs9470080 and rs9394309) that were also identified in the candidate gene study above. These SNPs, located in the *FKBP5* gene region, were associated with a lower $cortisol_{AUC}$ per risk allele (rs9470080: beta=-0.55, p-value 1.26 x 10(-5), rs9394309: beta=-0.56, p-value 1.58 x 10(-5)). The first 50 hits of the GWAS, the Q-Q plot and Manhattan plot are included in the SM (see (SM) table 3, fig. 1 and fig.2). The GWAS stratified on gender (776 males and 931 females) yielded less SNPs with p-values of 1x10(-5) or smaller than the GWAS in the whole sample. Two

Table 1 Characteristics of Rotterdam Study participants and Whitehall II Study participants with cortisol data.

	Rotterdam Study (n=1711)	**Whitehall II Study** (n=2836)	*p*-value[a]
Gender (male)	45.4%	77.7%	<0.001
Age (yrs.)	74.9(5.7)[b]	60.9 (5.9)[b]	<0.001
Cortisol (nmol/L)			
-at awakening	13.17(0.01-42.97)	14.80(0.10-40.05)	<0.001
-30min. after awakening	16.80(0.06-51.12)	21.82(0.01-54.52)	<0.001
-8h. after awakening	3.42(0.01-19.09	5.13(0.00-19.50)	<0.001
-at bedtime	1.64(0.02-13.42)	1.50(0.00-13.59)	0.005
$Cortisol_{AUC}$ (nmol/L)	8.14(3.47)[b]	6.93(2.29)[b]	<0.001

Numbers are median (100% range) unless otherwise indicated

[a] t test and Mann Whitney test for continuous variables and Chi-square tests for dichotomous variables.

[b] mean (standard deviation)

Table 2 Selected candidate genes expected to explain variation in cortisol secretion

Gene Symbol[a]	no. of SNPs (Illumina550k)	Position
Corticosteroid biosynthesis		
CYP21A2	35	6:32014061-32217398
CYP11B1	35	8:143850775-144058238
CYP17A1	35	10:104480278-104687280
HSD3B2	39	1:119659296-119867174
CYP11A1	48	15:72317156-72547134
MC2R	48	18:13774624-13975517
STAR	7	8:38019375-38227757
PRKAR1A	41	17:63923130-64159026
Central HPA-axis control & negative feedback		
NR3C1	58	5:142537689-142863447
NR3C2	104	4:149120957-149677462
AVP	45	20:2911203-3113370
AVPR1A	44	12:61726483-61932857
CRH	24	8:67,251,173- 67,253,252
CRHR1	47	17:41117449-41368973
CRHR2	53	7:30559388-30788665
POMC	34	2:25137226-25345063
MC4R	48	18:56089544-56290981
POU1F1	42	3:87291473-87508427
PROP1	26	5:177251842-177455849
TBX19	49	1:166416902-166650288
LEPR	82	1:65558906-65975410
LEP	37	7:127568567-127784918
FKBP5	48	6:35549346-35864692
Cortisol inactivation and metabolism		
HSD11B2	8	16:65922537-66128955
HSD11B1	58	1:207826173-208074918
DHRS9	32	2:169534209-169746650
SRD5A1	78	5:6586500-6822675
SRD5A2	20	2:31503160-31759544
AKR1D1	56	7:137327054-137553590
CYP3A4	20	7:99092540-99319744
H6PD	55	1:9117450-9353981
SERPINA6	73	14:93740338-93959441
ACE	26	17:58808166-59048711
33 genes	1456 SNPs	

Abbeviations: SNP, single nucleotide polymorphism

[a] full gene names are given in supplementary information (SI) table 1 available online

Table 3 The association of candidate gene SNPs with cortisol$_{AUC}$

SNP									
Most significant	No. Significant	Gene	Chr.	Variant	MAF	beta	se	*p*-value unadj.	*p*-value adj.[a]
cortisol$_{AUC}$ (nmol/L)									
rs9470080	4	FKBP5	6	G > A	0.34	-0.55	0.12	1.0e-05	0.001
rs11152221	-	MC4R	18	G > A	0.32	-0.36	0.12	3.1e-03	0.18
rs2284218	-	CRHR2	7	A > G	0.36	0.34	0.12	5.6e-03	0.35
rs2296236	-	AVP	20	G > A	0.16	-0.44	0.16	7.1e-03	0.40
rs7735244	-	SRD5A1	5	A > G	0.10	-0.50	0.19	9.6e-03	0.44
rs10519942	-	NR3C2	4	G > A	0.07	0.59	0.23	0.009	0.48
rs6672903	-	HSD3B2	1	A > G	0.44	0.30	0.12	0.012	0.59

Abbreviations: SNP, single nucleotide polymorphism; Chr., Chromosome; MAF, minor allele frequency; se, standard error

[a]adjusted for multiple testing using max (T) permutation (10.000).

top SNPs of the GWAS in the whole sample were also found among the top hits in the stratified analyses; males rs9470080 p-value 9.95x10(-5), females rs8026512 p-value 1.94x10(-5). The results of the stratified analyses are presented in table 4a (males) and table 4b (females) of the supplementary material.

To replicate our main findings, top hit SNPs (rs8026512, rs2252459, rs9470080 and rs9394309) were genotyped in the Whitehall II study.Of the four SNPs, two (rs8026512 and rs2252459) were associated with the cortisol$_{AUC}$ in the replication cohort; however, the effects were in the opposite direction. Hence, none of the initial SNPs associated with cortisol secretion in the GWAS sample were successfully replicated.

The association of candidate genes and clinically relevant depressive symptoms.

Table 5 presents the association between the 4 SNPs which remained significant after adjustment for multiple testing in the candidate gene analysis, and depressive symptoms. Since these SNPs are in strong LD, our results were not corrected for multiple testing. Carriers of minor alleles of rs9470080 were at an increased risk of depressive symptoms (OR1.19, 95%CI 1.01; 1.40, p-value 0.037).

The distribution of gender, age, educational level, smoking, DM type II, CHD and use of hypnotics did not differ between carriers of rs9470080 and non-carriers. In contrast, carriers of rs9470080 were more frequently using antidepressants or antipsychotics compared with non-carriers (SM table 5)

DISCUSSION

This study from two well-characterized European cohorts of middle-aged or elderly adults used candidate gene and genome wide approaches to identify genes associated with diurnal cortisol secretion and their association with depressive symptoms. We found evidence for an association of *FKBP5* SNP rs9470080 with both saliva cortisol concentrations and depressive symptoms.

FKBP5 is a co-chaperone of hsp90, which is part of a receptor complex that regulates the sensitivity of the glucocorticoid receptor (GR). An increase in FKBP5 gene expression leads to an increased resistance of GR to cortisol, which may result in hypercortisolism [24]. GWAS and linkage studies, one of which includes data from the Rotterdam Study, did not report SNPs within the FKBP5 gene as one of the most significant associations [25-27]. In previous candidate gene studies, *FKBP5* SNPs have been associated with depression [10-11], the recurrence of depressive episodes, the response to antidepressant treatmen[12, 28], peritraumatic dissociation in children [29], bipolar disorder [30], and suicide behaviour [31-32]. FKBP5 gene expression was associated with posttraumatic stress disorder (PTSD) [33]. Furthermore, carriers of FKBP5 SNPS showed insufficient recovery of cortisol levels after psychosocial stress [34]. Also, interaction effects have been found with FKBP5 SNPs and childhood abuse on PTSD [35] and FKBP5 SNPs and resistant attachment on cortisol reactivity in infants [36]. Most studies examined the effect of *FKBP5* SNPs under stressful circumstances only. Such a paradigm may activate different regulatory mechanisms than those regulating basal cortisol secretions.

In the present study, carriers of genetic variants in the *FKBP5* gene had a lower cortisol_{AUC} and an increased risk of depressive symptoms compared with non-carriers. The latter finding is in line with the observation that TT-carriers of the *FKBP5* rs1360780 variant are more likely to have recurrent and treatment-refractory depression. However, depression is typically associated with high cortisol levels. Several aspects, other than a chance finding, must be considered when interpreting these findings. Firstly, the relation between depression and hypercortisolism is less consistent than frequently stated. In a meta-analysis, Burke and colleagues showed that the association between depression and cortisol levels at baseline strongly depends on the time of day. In the morning, depressed patients show lower cortisol levels than non-depressed individuals. In the afternoon, cortisol levels are higher among depressed patients compared to non-depressed individuals. Also, Oldehinkel and colleagues found that hypocortisolism, and not hypercortisolism, was related to chronic depressive episodes [37]. Furthermore, Penninx and colleagues reported a U-shaped relation between depression and cortisol among elderly subjects. In this study, depressed elders with the lowest cortisol levels also scored higher on frailty indicators than depressed elders with cortisol levels in the highest tertile [38]. Furthermore, hypocortisolism has been associated with depression in elderly females [39]. This may explain the association of FKBP5 SNPs with lower corti-

Table 4 Main findings GWAS cortisol$_{AUC}$ and replication results

SNP	Gene	Chr.	Variant	Rotterdam Study (n=1711)		Whitehall II Study (n=2836)		Meta-analysis (n=4547)	
				beta	*p*-value	beta	*p*-value	beta	*p*-value
cortisolAUC (nmol/L)									
rs8026512	-	15	T > C	-0.57	6.04e-06	0.13	0.037	-0.01	0.830
rs2252459	ALCAM	3	T > C	0.53	8.75e-06	-0.13	0.039	0.01	0.803
rs9470080	FKBP5	6	G > A	-0.55	1.26e-05	-0.025	0.702	-0.14	0.017
rs9394309	FKBP5	6	A > G	-0.56	1.58e-05	-0.006	0.927	-0.12	0.037

Abbreviations: SNP, single nucleotide polymorphism; Chr., Chromosome

Table 5 Results of the association of FKBP5 SNPs with depressive symptoms (n=2361)

				CES-D Depressive symptoms score >/=16[a]			LD with rs9470080	
Chr.	SNP	Position	Variant	OR	95%CI	*p*-value	ΔR^2	D′
6	rs9470080	35754413	G>A	1.19	1.01;1.40	0.037	-	-
6	rs9394309	35729759	A>G	1.15	0.97;1.35	0.107	0.96	1
6	rs7748266	35700722	G>A	1.16	0.95;1.43	0.151	0.47	1
6	rs1360780	35715549	G>A	1.18	1.00;1.39	0.057	0.84	1

Abbreviations: CES-D, Centrum for Epidemiology Studies Depression Scale; LD, linkage disequilibrium; Chr, chromosome; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval

[a] reference = CES-D depressive symptoms score < 16

sol$_{AUC}$ and depression in our study, since our study of elderly was predominantly female. Second, depression in community-dwelling elderly is typically more chronic as a consequence of the ascertainment, but acute and clinical depression is characterized by high levels of cortisol. Third, we studied depressive symptoms and not a clinical diagnosis of depression or subtypes of depression. However, the observation that carriers of FKBP5 rs9470080 were more likely to report clinically relevant depressive symptoms and also more likely to use antidepressants or antipsychotics further indicates that depressive symptoms are a good marker of severe psychiatric disorders. Also, we did not rule out persons with depressive symptoms due to PTSD, fibromyalgia or chronic fatigue, which have been associated with hypocortisolism [40].Finally, the relation between *FKBP5* variants and depression could not be explained by the cortisol parameters in the present study as there was no association between cortisol$_{AUC}$ and depressive symptoms. This can reflect a lack of power, but could also indicate that another mechanism than basal cortisol regulation underlies the observed associations.

The GWAS approach identified two SNPs which are potentially relevant for cortisol secretion. Our top hit (rs8026512) was located on chromosome 15 in the Prader Willi

syndrome (PWS) deletion region. This syndrome is a neurobehavioural disorder characterized by hypertonia, failure to thrive, hyperphagia, short stature, hypogonadism and developmental delay. De Lind van Wijngaarden and colleagues [41] reported a 60% prevalence of central adrenal insufficiency in PWS patients, which is characterized by insufficient cortisol. The second hit (rs2252459) is located in intron 3 of the activated leukocyte cell adhesion molecule (ALCAM) gene on chromosome 3 (3q13.1). This cell adhesion molecule is expressed by epithelial cells in several organs. Alterations in the expression of ALCAM have been reported in several human tumours [42]. Although it is not directly clear how ALCAM may influence cortisol secretion, there may be a link between ALCAM and cortisol via the immune system.

The replication of the association of four SNPs with cortisol$_{AUC}$ from the GWAS in the Rotterdam Study (RS) was not successful in the Whitehall II study. The associations found in the replication sample were not in the same direction and had thus not been hypothesized based on the initial GWAS. They were also not significant after Bonferroni correction.

Inconsistent findings may be the result of false-positive findings in the GWAS, or falsely non-replicated true GWAS findings in the replication sample [43]. Due to the number of tests performed in a GWAS, the frequency of false-positive findings is high. In this study with 1711 participants, we had sufficient power (0.80) to detect an R^2 change of 0.0045 (Quanto 1.2.4, [44-45]). We had 0.55 power to detect the effect sizes (beta -0.56) associated with our strongest hits. Furthermore, phenotyping and genotyping errors may account for false-positive findings in a GWAS. . However, in both studies cortisol data were obtained by saliva sampling at home with several samples on one day, and cortisol levels were assessed in the same laboratory. Albeit statistically significant, the differences in cortisol levels were small. Hence, the phenotype definition is unlikely to account for the non-replication. The other differences between the two studies are likely the results of the difference in inclusion criteria of both studies. The WHII Study consists of civil servants from London which implies that the study population has, on average, a high educational level and more men. This high social economic status may account for differences in the prevalence of disease, and the use of hypnotics. It has been reported that cortisol levels increase with age [46-47] Also, there is evidence to suggest that cortisol levels are higher in older men than older women [46]. Grant and colleagues reported higher urinary free cortisol levels in depressed men than in depressed women. These urinary free cortisol levels in depressed males further increased with age and severity of the illness [48]. As the participants of the Rotterdam Study and the Whitehall II Study were all older adults, it seems unlikely that difference in age accounts for the non-replication. Gender distribution in the two cohorts was different with a majority of female participants in the Rotterdam Study and a majority of males in the replication sample. However, the GWAS of cortisol$_{AUC}$ stratified on gender did not yield associations with lower p-values, which is likely due to insufficient power. The GWAS and the replica-

tion study were not adjusted for other covariates related to cortisol levels, since SNP associations are very unlikely to be confounded by these variables. This is because most confounding factors do not alter an individual's genetic make-up. Adjusting for covariates that affect cortisol secretion may increase precision of our phenotype, but could also blunt the measured gene-phenotype association [49].

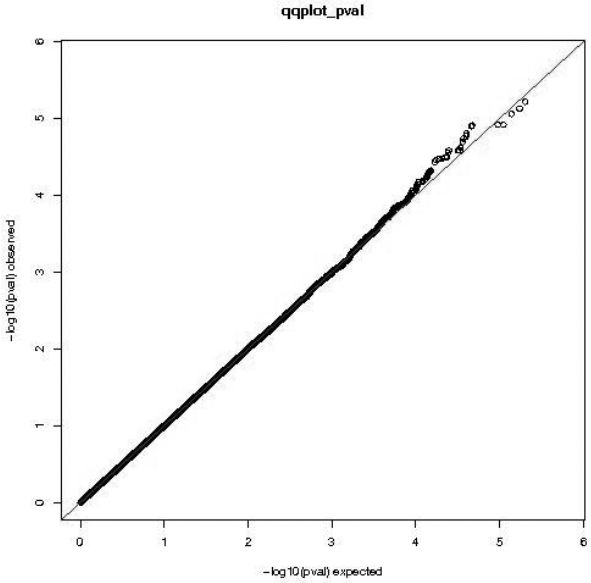
Next to cortisol$_{AUC}$, the single cortisol measurements and the cortisol awakening response (CAR) are frequently used phenotypes of cortisol secretion. In this study, we focused on the cortisol$_{AUC}$, because this daytime profile gives more information about the HPA-axis activity than the single cortisol measurements and reduces the risk of multiple testing [15]. The CAR was not used as outcome measurement, because its exact function remains to be determined and conflicting results, both an elevated CAR as well as a blunted CAR, in relation to depression have been found [50].

Next to false-positive findings, it could be that our GWAS findings were falsely non-replicated in the WHII study. This may be due to phenotyping and genotyping errors or low power. As discussed previously, the phenotyping in the two samples was very comparable. Also, all SNPs were in HWE and there was no miscoding of major versus minor alleles. To improve power, replication studies with substantially larger sample sizes are needed to reliably determine whether we observed true associations or chance findings in the GWAS of cortisol$_{AUC}$.

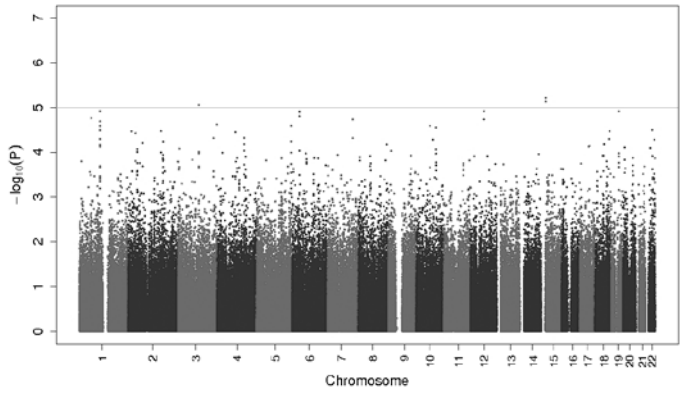
At least two other studies have used a genome wide approach to identify genes associated with cortisol secretion. Ukkola and colleagues performed a genome wide linkage scan on morning serum cortisol in black and white families, but did not find evidence for linkage between any of the microsatellite markers and cortisol [51]. A subsequent family-based linkage study identified a significant association between morning serum cortisol and two markers on chromosome 11 and 14 in women [52]. Although saliva cortisol levels and serum cortisol levels are highly correlated [13-14], morning levels of cortisol tend to be highly variable due to the steep morning rise and do not represent cortisol$_{AUC}$ well. Therefore, the lack of power to identify complex gene variants using the linkage approach, and the difference in phenotype definition are likely to account for the lack of congruence between these two studies and our study.

In conclusion, we found an association of *FKBP5* SNPs with a decrease in cortisol$_{AUC}$ in a population-based cohort. Carriers of *FKBP5* minor alleles were also at increased risk of clinically relevant depressive symptoms. Although this is consistent evidence for the physiological and clinical relevance of variants in this HPA-axis regulating gene in an epidemiological study, future laboratory studies are needed to establish the causal mechanism behind these associations.

SUPPLEMENTARY MATERIAL



SM Figure 1 Quantile-quantile plot of the genome-wide-association study of cortisol$_{AUC}$. *P*-values are obtained from linear regression using the additive genetic model and were plotted on a logarithmic scale.



SM Figure 2 Manhattan plot of the *p*-values in the genome-wide-association study of cortisol$_{AUC}$. *P*-values are obtained from linear regression using the additive genetic model and were plotted on a logarithmic scale. The x-axis represents chromosomal location and the y-axis shows *p*-values on a logarithmic scale. The grey horizontal line indicates a *p*-value of 1x10(-05).

Supplementary Material table 3. The top 50 associations of the GWAS on cortisol$_{AUC}$ (nmol/L).

Chromosome	SNP	Position	BETA	SE	unadj. *p*-value
15	8026512	21808752	-0,57	0,13	6,04E-06
15	11630255	21800234	-0,57	0,13	7,45E-06
3	2252459	106729462	0,53	0,12	8,75E-06
1	7552405	104915372	-0,56	0,13	1,21E-05
12	983665	65576010	0,97	0,22	1,21E-05
19	520194	39383289	0,55	0,13	1,23E-05
6	9470080	35754413	-0,55	0,13	1,26E-05
6	9394309	35729759	-0,56	0,13	1,58E-05
1	4915695	60734756	0,78	0,18	1,70E-05
7	17164729	129629465	-0,96	0,22	1,82E-05
12	924822	65580728	1,03	0,24	1,82E-05
1	2121956	104890919	0,52	0,12	2,03E-05
3	10933645	196655709	0,61	0,14	2,43E-05
5	2936953	173738680	-0,53	0,13	2,60E-05
10	766031	67347745	0,58	0,14	2,60E-05
1	6695172	104887574	0,51	0,12	2,61E-05
10	12255572	97318217	-1,31	0,31	2,80E-05
22	2267298	32554916	0,49	0,12	3,16E-05
1	11184197	104883296	0,5	0,12	3,22E-05
2	4664397	161610194	-0,58	0,14	3,32E-05
18	1866732	71062175	-0,52	0,13	3,48E-05
2	7571070	14251646	-0,56	0,13	3,36E-05
4	10516756	86923000	1,03	0,25	3,50E-05
2	6719824	33457552	-0,98	0,24	3,69E-05
4	2085679	131715156	0,51	0,13	4,74E-05
7	10241132	129587081	-0,73	0,18	4,84E-05
3	10936959	179073768	-0,54	0,13	4,85E-05
18	4430852	68043724	0,59	0,15	5,08E-05
1	12407649	104600376	-0,49	0,12	5,10E-05
10	4934419	82444275	0,51	0,13	5,23E-05
22	1894524	43681326	0,61	0,15	5,30E-04
5	718927	173771766	0,49	0,12	5,71E-05
2	218311	171766110	0,5	0,12	5,72E-05
2	4464317	69290795	0,48	0,12	6,17E-05
4	2311375	131721644	0,5	0,13	6,51E-05
18	6507842	44141990	0,52	0,13	6,58E-05
8	13259162	141279396	0,51	0,13	6,65E-05

1	12568560	104941822	-0,56	0,14	6,81E-05
17	2628302	50513360	-0,46	0,12	7,14E-05
1	10785866	104688782	-0,52	0,13	7,53E-05
17	2215290	46579220	0,76	0,19	7,58E-05
20	6054292	6402661	0,56	0,14	7,84E-05
22	915611	23425452	-0,49	0,12	8,09E-05
3	9859247	8005809	-0,51	0,13	8,45E-05
2	3765000	43867805	0,86	0,22	8,58E-05
10	10882635	97338835	-1,11	0,28	8,63E-05
6	7748266	35700722	-0,65	0,17	9,23E-05
4	1968821	131718847	0,55	0,14	9,31E-05
9	12002454	14537633	0,86	0,22	9,36E-05
3	4894924	106595927	-0,46	0,12	9,81E-05
2	4952679	43883325	0,84	0,22	9,82E-05

Abbreviations: SNP; single nucleotide polymorphism, se; standard error

Supplementary material table 4a

Top 20 hits GWAS cortisol$_{AUC}$ in males of the Rotterdam Study.

SNP	**Gene**	**Chr.**	**Variant**	**Rotterdam Study Males n=776**	
cortisol$_{AUC}$ (nmol/L)			beta	*p*-value	
rs7248997	-	19	A>G	0.9043	2.76e-06
rs9534093	FRY	13	G>T	-2.3640	5.75e-06
rs17061204	MOXD1	6	T>G	3.3790	7.01e-06
rs6509933	-	19	T>C	0.7990	1.33e-05
rs973753	PRKCA	17	T>C	1.0540	1.42e-05
rs4790911	PRKCA	17	G>A	1.3390	1.80e-05
rs10214527	-	6	G>T	0.8198	2.00e-05
rs7233804	LOC100127987	18	G>A	0.9588	2.01e-05
rs4417581	PRKCA	17	C>A	1.0980	2.54e-05
rs6049635	ZNF343	20	T>C	-0.7326	2.74e-05
rs2963790	-	5	A>G	0.7709	3.61e-05
rs1659025	-	13	G>A	0.8016	3.80e-05
rs9849665	-	3	G>A	-0.7218	4.39e-05
rs1708479 / rs1084722	-	3	T>C	-0.7218	4.39e-05
rs2310001	ENPP6	4	T>C	0.7698	4.43e-05
rs1458642	-	4	T>G	-0.7759	4.61e-05
rs1408095	-	X	C>T	1.1040	5.46e-05
rs1854779	IVL	1	A>G	1.1870	5.56e-05
rs717207	KCNMA1	10	G>T	-1.5740	6.15e-05

Abbreviations: SNP, single nucleotide polymorphism; Chr., Chromosome

Supplementary material table 4b

Top 20 hits GWAS cortisol$_{AUC}$ in females of the Rotterdam Study.

SNP	Gene	Chr.	Variant	Rotterdam Study Females n=931	
cortisol$_{AUC}$ (nmol/L)			beta	*p*-value	
rs2949574	-	15	A>G	0.7822	1.04e-6
rs7105794	ODZ4	11	G>T	0.8396	3.27e-6
rs8020438	-	14	T>G	1.4630	3.30e-6
rs8020822	-	14	T>C	1.4370	5.62e-6
rs2206860	-	6	T>C	-0.7149	7.09e-6
rs7087051	TYSND1	10	G>A	1.5860	7.61e-6
rs2237364	CREB5	7	G>T	-0.7300	7.74e-6
rs4779794	FAN1	15	G>A	0.6945	1.03e-5
rs17550045	STON2	14	C>A	-1.1590	1.15e-5
rs542931	SLC35F2	11	T>C	0.7231	1.16e-5
rs2075678	CSE1L	20	A>G	-0.7053	1.16e-5
rs16914695	-	9	G>A	0.8300	1.50e-5
rs11055930	-	12	G>A	-0.7463	1.54e-5
rs2293314	FAN1	15	T>C	0.6728	1.76e-5
rs1968821	-	4	T>C	0.8257	1.83e-5
rs2955790	-	15	T>G	0.6693	1.94e-5
rs8026512	-	15	C>T	-0.7128	1.94e-5
rs3091529	ARFGEF2	20	A>G	1.1870	1.95e-5
rs10969210	-	9	G>T	-1.5740	1.96e-5

Abbreviations: SNP, single nucleotide polymorphism; Chr., Chromosome

Supplementary material table 5
Characteristics of *FKBP5* rs9470080 non-carriers and carriers of the T allele.

	rs9470080				
	CC	CT	TT	Test statistic*	*p*-value
Gender male (%)	41.5	40.3	42.3	0.675	0.714
Age in years mean(sd)	75.4 (6.0)	75.8 (6.4)	76.2 (6.5)	2.205**	0.110
Education (%) University/ polytechnic					
Smoking (%)	15.9	13.2	14.8	5.588	0.232
CHD (%)	14.1	12.6	12.3	1.468	0.480
DM type II (%)	6.6	6.0	5.6	3.655	0.455
Antidepressants/ Antipsychotics (%)	4.3	5.6	7.8	6.210	0.045
Hypnotics (%)	16.3	16.5	20.5	3.358	0.187

*chi-square unless otherwise indicated

** ANOVA

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Chapter 4

Gene-environment interaction;
the Glucocorticoid Receptor
gene and the FKBP5 gene.

Chapter 4.1

FKBP5 and Resistant Attachment Predict Cortisol Reactivity in Infants: Gene-Environment Interaction

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ABSTRACT

Quality of the parent-infant attachment relationship influences physiological stress regulation. Genetic factors also contribute to the stress regulatory HPA-axis. Quality of attachment as an index of the rearing environment (measured with the Strange Situation Procedure, SSP), and HPA-axis related SNPs (*Bcl*I, rs41423247; *TthIII*I, rs10052957; GR-9β, rs6198; N363S, rs6195; ER22/23EK, rs6189 and 6190; and FKBP5, rs1360780) were hypothesized to be related to cortisol reactivity in the stressful SSP. In this large population based sample, FKBP5 rs1360780, but not GR haplotype, was related to cortisol reactivity. Moreover, we found a significant interaction effect for insecure-resistant attachment and FKBP5 rs1360780, indicating a double risk for heightened cortisol reactivity levels in infants with one or two T-alleles of the FKBP5 SNP *and* an insecure-resistant attachment relationship with their mother. Findings are discussed from the perspective of gene-environment interaction.

INTRODUCTION

The infant-parent attachment relationship plays a major role in the infant's early life, particularly for socio-emotional development and emotion regulation [1-2]. The quality of the attachment relationship not only influences regulation on the behavioral level, but also affects physiological regulation. The physiological system is activated in stressful contexts, especially when coping behaviors are inadequate or coping resources are unavailable [3]. Most studies on the physiology of infant attachment relationships focused on measures of heart rate and cortisol during the Strange Situation Procedure (SSP) [4-7], a mildly stressful procedure with two brief separations from the caregiver in an unfamiliar environment. Differences in physiology during this procedure have been predominantly attributed to the quality of attachment which is an index of the rearing environment. Genetic factors have not received much attention, although there is ample evidence that genetic factors play a role in explaining variance in HPA-axis activity [8-10]. In the current study, both quality of attachment and genetic variations associated with HPA-axis activity were examined in relation to cortisol reactivity. In addition, the interaction between genetic factors and attachment quality on cortisol reactivity was investigated.

Studies on the association between attachment quality and cortisol levels have focused mainly on stress reactivity, with assessment of cortisol levels before and after the stressful SSP. The SSP is the gold-standard procedure to assess the quality of the infant-caregiver attachment relationship. The SSP allows for classification of the relationship as secure, insecure-avoidant, or insecure-resistant. Securely attached (B) children seek contact with the parent upon reunion, either physically or by distance interaction, to be comforted or reassured after the separation and resume exploration of the environment when they are settled. Based on their interactions with the caregiver, they have learned that she/he is available in times of stress. In contrast, infants of inconsistently sensitive or consistently insensitive parents do not come to expect their parents to be available in stressful situations, with insecure (avoidant or resistant) attachment relationships as a result. Children with insecure-avoidant (A) attachments focus on the environment at the moment of reunion, ignoring the parent or even turning away from them. The reunion behavior of an insecure-resistant (C) child is characterized by anxious contact seeking and clinging and at the same time resisting contact with the parent. On top of these classifications, attachment disorganization can be observed and rated. Disorganized (D) children show a temporary breakdown of their secure, avoidant or resistant strategy of dealing with the return of the parent after separation [11].

In several non-clinical studies, children tended to show elevated cortisol levels in reaction to the SSP. The most consistent finding is that only little or no adrenocortical activation is observed in securely attached infants, and increased cortisol levels for the

disorganized infants [3, 12-13]. Results for the insecure-avoidant and insecure-resistant groups are inconsistent. In some studies, both insecure groups were found to have elevated cortisol levels after the SSP [13], others found increased cortisol levels only for insecure-resistant children [14]. In a previous study on the current sample [15], we found increased cortisol levels for insecure-resistant children.

Associations between attachment quality and cortisol reactivity implicate that cortisol reactivity levels are, at least partly, determined by the caregiving environment [16-17]. Evidence for the contribution of genetic factors has been mixed [9-10, 18] and it has been noted that 'the genetic and environmental contributions to cortisol reactivity in early childhood have yet to be documented' [18]. This interplay between genetic and environmental factors was recently studied by Frigerio and colleagues [19], who found independent effects of candidate genes (5HTT, GABRA6, DRD4, and COMT) and attachment quality on alpha amylase, another potential biomarker for physiological arousal. They did not, however, find effects of attachment quality, genetics, or their interaction on cortisol reactivity. In view of these diverging findings, they note that replication in larger samples is required.

Recently, specific candidate genes that play a role in explaining variability in cortisol reactivity have been identified. Several studies focused on the glucocorticoid receptor (GR) that mediates many of the effects of glucocorticoids. Genetic variants of the GR gene, e.g. single nucleotide polymorphisms (SNPs), appear to contribute to interindividual variability in HPA-axis activity by affecting a cell's sensitivity for glucocorticoids [20-21]. Five different SNPs within the GR have been investigated in previous research; *Bcl*I (rs41423247), *TthIII*I (rs10052957), GR-9β (rs6198), N363S (rs6195) and ER22/23EK (rs6189 and 6190). No effects of GR on basal cortisol excretion have been found [22-23]. However, HPA-axis reactivity as assessed using a social stressor, the Trier Social Stress Test, showed that carriers of the N363S G allele had increased cortisol responses. On the other hand, carriers of the *Bcl*I G allele and GR-9β G allele showed an attenuated response [21, 24-25].

Importantly, blocks of specific SNP combinations are usually found within genes, resulting in several haplotypes, that is, groups of specific SNPs in a gene that tend to be inherited together. These haplotypes can have different effects compared to 'isolated' SNPs [20]. In the current study, effects of GR haplotypes on cortisol reactivity will be tested [26-27]. GR activation is regulated by a large molecular complex. In this complex, several molecules, so-called chaperones and co-chaperones, play a critical role. Altering the composition of the (co-)chaperones influences sensitivity of GR to cortisol and thus affects HPA-axis responsivity [28-29]. The FKBP5 co-chaperone of GR has been associated with changes in HPA-axis activity by altering the negative feedback system [24]. The feedback loop is crucial in recovery from stress, which in turn is essential for healthy physiological and behavioral regulation. As the infant-parent attachment relationship

can be considered the infant's most important emotion regulation system [1-2], the role of a genetic factor influencing homeostasis might be of great importance. FKBP5 has several SNPs, and for these SNPs the most consistent findings were reported for rs1360780. For individuals carrying one or two copies of the minor (T) allele, i.e. the allele that is less frequent in the population, positive associations have been found with major depression, bipolar disorder, post-traumatic stress disorder and a faster response to antidepressant treatment (for a review, see [28]). With respect to HPA-axis activity, this SNP did not show an effect on basal cortisol levels [29], but it did show an effect on cortisol responses to the Trier Social Stress Test [24]. Participants who were homozygous for the minor allele (TT genotype) showed an impaired recovery from stress compared to carriers of the CC or CT genotype.

In the current study we expand the findings on attachment security and cortisol reactivity from previous studies [3, 12, 14] by adding a genetic component. Carriers of the minor alleles of the haplotypes of GR and the FKBP5 SNP were expected to show altered cortisol reactivity levels. Furthermore, it is hypothesized that the association between attachment security and stress reactivity is moderated by GR and FKBP5. A combination of insecure-resistant attachment and carrying one or more 'risk alleles' of GR and the FKBP5 SNP was expected to lead to higher cortisol reactivity.

METHOD

Setting

The current investigation is embedded within the Generation R Study, a prospective cohort study investigating growth, development and health from fetal life into young adulthood in Rotterdam, the Netherlands, which has been described in detail elsewhere [30-31]. In the Generation R Study, we obtained detailed measurements of the child's development in a rather homogeneous subgroup: The Generation R Focus Study. Only children of Dutch national origin were included in this group, meaning that the children, their parents and their grandparents were all born in the Netherlands. The participating children were born between February 2003 and August 2005. The children visited the research center regularly for various somatic and behavioral assessments. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

Study Population

DNA was collected from cord blood samples at birth. At the age of 14 months, infants and their mothers participated in the Strange Situation Procedure (SSP). In 589 infants, information on GR and FKBP5 genotypes and quality of attachment was available. Of

this group, cortisol was sampled in 310 children. Unsuccessful sampling was mainly due to refusal to chew on cotton swabs, which is not uncommon in this age group and has been reported before [32]. This is typically found in infants that are not familiar with pacifiers. A non-response analysis was conducted to check for differences between children with and without cortisol data. Differences between the groups were found for gender ($p = .01$); the group with cortisol data consisted of more boys. Also, educational level of the mother differed ($p = .03$); mothers in the group for which data was available were less highly educated. No differences were found in the distribution of attachment classifications or genotype ($.09 < p < .87$).

Procedures and Measures

Strange Situation Procedure. Parent-infant dyads were observed in the Strange Situation Procedure [6] when the infant was about 14 months of age (M = 14.7 SD = 0.9). The SSP is a widely used and well-validated procedure to measure the quality of the attachment relationship. The procedure consists of seven episodes of 3 minutes each and is designed to evoke mild stress in the infant to trigger attachment behavior evoked by the unfamiliar lab environment, a female stranger entering the room and engaging with the infant, and the parent leaving the room twice (see Ainsworth et al., 1978, for the protocol). The SSP used in the current study included all these stimuli but to make it fit into a tight time schedule, we shortened the (pre-) separation episodes with one minute keeping the critical reunion episodes intact. Attachment behavior was coded from DVD-recordings according to the Ainsworth et al. (1978) and Main and Solomon (1990) coding systems by two reliable coders, trained at the University of Minnesota. Inter-coder agreement was calculated on 70 SSPs that were coded by both coders. For ABCD classification, inter-coder agreement was 77% ($\kappa = .63$); agreement on disorganization was 87% ($\kappa = .64$). Eight percent of the cases were discussed with one of two expert coders and classification was assigned after consensus was reached.

Salivary cortisol: stress reactivity. During the visit at the research centre at 14 months of age, three saliva samples were taken using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany); the first prior to the SSP, the second directly after the SSP (which was on average 10 minutes after the first separation of the SSP) and the third about 15 minutes later ($M = 16.3$, $SD = 8.3$). None of the children used systemic corticosteroid medication, but 12 children used other corticosteroid-containing medication. Excluding these children did not change the results, thus we included them in further analyses.

Samples were centrifuged and frozen at -80°C. After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the Kirschbaum laboratory (Technical University of Dresden, Biological Psychology, Professor Dr. Kirschbaum) for analysis. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and

interassay coefficients of variation were below 7% and 9%, respectively. For each time point, cortisol values that were above the 99th percentile (>200 nmol/L) were excluded (n = 12) from the analysis to reduce the impact of outliers.

Cortisol analyses. For stress reactivity a delta was calculated between the last sample ($cortisol_{postSSP}$) and the first sample ($cortisol_{preSSP}$). The second assessment, just after the SSP, was not used, as it was too close to the onset of stress. To control for the Law of Initial Values [33], which states that the direction of response of a body function depends to a large degree on the initial level of that function, in subsequent analyses this delta was adjusted for the first sample.

Genotyping. DNA was collected from cord blood samples at birth. To check for potential contamination with maternal blood, gender was determined in male participants. Contamination occurred in < 1% of cases, which were excluded. All participants were genotyped for polymorphisms in the glucocorticoid receptor gene, *Bcl*I (rs41423247), *TthIII*I (rs10052957), GR-9β (rs6198), N363S (rs6195) and ER22/23EK (rs6189 and 6190); and the FKBP5 gene (rs1360780). Table 1 shows the allele frequencies for the GR SNPs. Genotyping was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 min), then 40 cycles of 94° C (15 s) and 60° C (1 min)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 97-99% of the samples. To confirm the accuracy of the genotyping results 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1% for all genotypes. For the glucocorticoid receptor gene we used the genotype data for each of the 5 polymorphisms to infer the haplotypes present in the population using the program PHASE, which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (Stephens et al., 2001). For each haplotype, 3 genotype combinations were distinguished as carrying 0, 1, or 2 copies of the haplotype allele. Haplotype 1 carries the major alleles of the polymorphisms; therefore, the reference allele is defined as carrying 2 copies of haplotype 1. The FKBP5 SNP was presented in a similar way; frequency of the minor allele was indicated (0, 1 or 2 copies). Distribution for FKBP5 was as follows: 147 CC (47.4%), 139 CT (44.8%), 24 TT (7.7%). Table 2 shows the specific nucleotide variations and distribution of the GR haplotypes and FKBP5. Genotype frequencies were in Hardy Weinberg equilibrium (χ^2s [1, N = 310] < 1.28, ps > .26). GR haplotypes and the FKBP5 SNP were not correlated.

Statistical analyses. First, we checked whether demographic variables were related to cortisol, genotype, and attachment classification using ANOVAs and Chi-square tests. An ANCOVA was performed to test the association between attachment quality and

Table 1 Allele frequencies and minor allele frequencies for GR SNPs

GR SNP	Allele frequency (%)[a]			MAF (%)
	0	1	2	
*Bcl*I (rs4142347)	41	46	13	36
*TthIII*I (rs10052957)	46	44	10	32
GR-9β (rs6198)	68	29	3	18
N363S (rs6195)	91	9	0	4
ER22/23EK (rs6189/6190)	92	8	0	4

[a] % of copies of the minor allele. MAF = minor allele frequency. All GR SNPs were in HWE (χ^2s [1, N = 310] < 0.66, *p*s > .42).

Table 2 Distribution of GR haplotypes and FKBP5 and main effects on cortisol reactivity

GR haplotype	Nucleotide variations					Haplotype copies (%)			HF (%)	*B* (95% *CI*)	*p*- value
						0	1	2			
Wildtype	G	G	A	C	A	33	49	18	42	0.12 (-4.29; 4.54)	.96
*Bcl*I	G	G	A	**G**	A	62	33	5	22	0.49 (-3.98; 4.97)	.83
*TthIII*I + *Bcl*I	**A**	G	A	**G**	A	72	27	1	14	0.68 (-3.89; 5.24)	.77
GR-9β + *TthIII*I	**A**	G	A	C	**G**	75	24	1	13	1.07 (-3.49; 5.64)	.64
N363S	G	G	**G**	C	A	91	9	0	4	1.46 (-3.51; 6.43)	.56
ER22/23EK + GR-9β + *TthIII*I	**A**	**A**	A	C	**G**	92	8	0	4	-0.36 (-5.37; 4.66)	.89
FKBP5						Genotype frequency (%)			MAF (%)		
						CC	CT	TT			
rs1360780				C/T		47	45	8	30	1.28 (0.31; 2.25)	.01

Note. The nucleic acid changes are indicated in bold; C = Cytidine, G = Guanine, A = Adenine, T = Thymine. FKBP5 SNP was in HWE (χ^2 [1, N = 310] = 1.28, p = .26). MAF = minor allele frequency, HF = haplotype frequency.

cortisol reactivity levels, controlling for initial cortisol values. Because attachment security and attachment disorganization are considered orthogonal constructs [34], they were entered as two separate factors. The relation between attachment quality and genotypes was tested using a Chi-square test. A regression analysis was used to test the main effects of genotypes on cortisol reactivity, correcting for initial cortisol values. Associations were also tested for individual GR SNPs, which yielded similar results (data available upon request). Using a regression analysis, we tested for a potential interaction effect of insecure-resistant attachment, FKBP5, and GR on cortisol stress reactivity. For this analysis, infants with an insecure-resistant attachment classification were contrasted

to infants with a non-resistant classification. In the first step, the first cortisol assessment ($cortisol_{preSSP}$) was entered to control for initial values. In the second step, resistant versus non-resistant attachment classification was entered. In the third step, GR haplotypes and FKBP5 were entered. In the fourth step, interactions between GR haplotypes, and FKBP5 with resistant attachment classification were entered. Except for the first cortisol assessment, all variables were centered based on the N for which cortisol reactivity data was available.

RESULTS

Distribution of attachment

In the group for which both cortisol reactivity data was available ($N = 310$), distribution of attachment classifications was as follows: 56.7% secure ($n = 174$), 18.6% insecure-avoidant ($n = 57$), 24.8% insecure-resistant ($n = 76$). Of all children, 18.4% were classified as disorganized ($n = 57$), 81.6% were non-disorganized ($n = 253$), a common distribution in non-clinical populations [34]. Participant characteristics are displayed in Table 3. Time of cortisol assessment was not related to cortisol measures or attachment classification, in fact, none of the demographic variables were related to cortisol, genotype, and attachment classification at the same time.

Attachment quality, HPA-axis genes and cortisol reactivity

Infants with an insecure-resistant attachment relationship showed the highest cortisol reactivity levels from pre SSP to post SSP (F (2, 300) = 17.60, $p < .01$, $\eta^2 = .11$, see Table 4). We did not find significant differences in stress reactivity between the disorganized group and the non-disorganized group, nor an interaction effect of attachment security and attachment disorganization. Attachment quality was not related to glucocorticoid haplotypes ($.25 < p < .97$) or the FKBP5 SNP (rs1360780) ($p = .14$). Haplotypes of the glucocorticoid receptor were not related to cortisol reactivity ($.56 < p < .96$). The FKBP5 SNP was however related to cortisol reactivity ($B = .13$, $CI = 0.31; 2.25$, $p = .01$, see Table 1), indicating that infants with FKBP5-CT and TT genotypes showed increased cortisol reactivity. The more T alleles infants carried, the stronger was their cortisol reactivity. Using a regression analysis, we tested for a potential interaction effect of insecure-resistant attachment, FKBP5, and GR haplotypes on cortisol stress reactivity. Main effects of GR haplotypes ($.14 < p < .91$) and interactions between GR haplotypes and resistant attachment did not reach significance ($.32 < p < .85$). In Table 5, the most parsimonious model is presented. Main effects for resistant attachment and FKBP5 were significant ($\beta = .30$, $p < .001$; $\beta = .19$, $p < .001$, respectively), as was the interaction between FKBP5 and resistant attachment ($\beta = .12$, $p < .05$). The model explained 32% of the variance. Infants with

Table 3 Sample characteristics (N=310)

Child characteristics	
Child gender, % girls	43.5
Parity, % firstborn	61.9
Birth weight in grams	3543 (483)
Age at 14 months visit	14.6 (0.8)
Time of assessment cortisol prior to SSP	12:07 (2:00)
Parental characteristics	
Age at intake mother	31.7 (4.1)
Maternal educational level, % low/medium	39.9
Hours working, mother	28.2 (12.9)
Marital status, % single	5.6
Smoking during pregnancy, %	11.0
Alcohol during pregnancy, %	55.2
Breastfeeding at 6 months, %	31.0

Note. Unless otherwise indicated, values are mean (SD).

Table 4 Cortisol values pre SSP, post SSP and cortisol reactivity Δ (nmol/L)

	Cortisol pre SSP	Cortisol post SSP	Cortisol reactivity Δ[a]	
Secure	6.26 (5.06)	6.15 (4.25)	-1.21 (0.87)	
Insecure-avoidant	6.27 (5.04)	5.42 (3.60)	-0.61 (0.62)	
Insecure-resistant	5.89 (4.97)	9.92 (8.91)	4.04 (0.65)	**
Disorganized	6.27 (7.02)	7.08 (8.09)	1.07 (0.37)	
Non-disorganized	6.15 (4.44)	6.89 (5.29)	0.41 (0.74)	

Note. Unless otherwise indicated, values are M (SD). Cortisol reactivity Δ corrected for initial cortisol values. [a]Values are M (SE). ** F (2, 300) = 17.60, $p < .01$

Table 5 Regression analysis predicting cortisol reactivity from FKBP5 and insecure-resistant attachment, controlling for initial cortisol values

	B (95% *CI*)	β	*P*	F_{change}	R^2	R^2_{change}
Step 1				75.48	0.20	0.20
$Cortisol_{preSSP}$	-0.51 (-0.63;-0.40)	-0.42	<.001			
Step 2				36.52	0.28	0.09
Resistant attachment	2.10 (1.44; 2.76)	0.30	<.001			
Step 3				8.10	0.30	0.02
FKBP5	1.88 (0.85; 2.90)	0.19	<.001			
Step 4				5.29	0.32	0.01
FKBP5 * Resistant attachment	1.20 (0.17; 2.22)	0.12	.022			

Note. $R^2 = .32$. Final model F (4, 302) = 34.72, $p < .01$. β is a standardized coefficient and denotes SD change in cortisol reactivity per SD change in the predictor. The statistics are derived from the final block of the regression model.

a resistant attachment relationship and the FKBP5-CT genotype showed more increased cortisol reactivity than resistant infants with the CC genotype, and resistant infants with the FKBP5-TT genotype showed the largest increases in cortisol reactivity.

DISCUSSION

An insecure-resistant attachment relationship predisposes infants to heightened cortisol reactivity levels. Also, the minor allele of the FKBP5 SNP was associated with cortisol reactivity in an additive fashion; the more T alleles, the higher levels of cortisol reactivity. Furthermore, an interaction between insecure-resistant attachment and FKBP5 was found. This represents a double risk for heightened cortisol reactivity levels in infants who carry one or two T-alleles of the FKBP5 SNP and at the same time have an insecure-resistant attachment relationship with their mother.

Insecure-resistantly attached infants have been found to display high cortisol levels after a stressful stimulus in some studies [14], but not in others [7, 35]. Resistant infants' high activation of the attachment system may not be terminated soon after the reunion with the caregiver because they are unable to use the attachment figure effectively, which makes it difficult for these children to find a state of homeostasis [36]. In the current study, no effects of disorganization on stress reactivity were found. In a previous study on the same sample [15], we found evidence for an association between disorganized attachment and flattened cortisol diurnal rhythm, which may indicate a different stress mechanism in the disorganized group. As determinants of attachment disorganization differ from those of attachment security, genetic susceptibility as well as physiological and behavioral developmental outcomes might be only partly overlapping.FKBP5 rs1360780 has been associated with altered stress reactivity in an adult sample [24]; individuals homozygous for the minor allele (TT genotype) showed an impaired recovery from stress. Furthermore, Binder et al. [37] found that the FKBP5 SNP moderated the relation between child abuse and adult post traumatic stress disorder (PTSD), and alterations in attachment quality or HPA-axis sensitivity were suggested as possible mechanisms for this association. Combined with findings from the current study, evidence grows for the contribution of this minor allele to differences in GR sensitivity, and to differential activation of the feedback loop of the HPA axis when confronted by a psychological stressor [28]. The negative feedback system is essential in recovery from stressful situations, for example the SPP. A balanced stress recovery system that promotes homeostasis is of great importance. Presumably, both the minor allele of the FKBP5 SNP and an insecure-resistant attachment relationship prevent adequate termination of the stress reaction. This lack of homeostasis could put the child at developmental risk; long-term negative outcomes have been shown for both insecure attachment [38-39] and, indirectly, for carriers of the FKBP5 SNP [37]. It should be noted however, that associations in the current study are correlational, and that the underlying mechanisms need further elaboration.

In the current study GR haplotypes were not related to cortisol reactivity. However, these results should be interpreted with some caution. Whereas we had sufficient power to analyze the two most frequent haplotypes, the haplotypes including the SNPs N363S

and ER22/23EK displayed very low frequencies in the current sample. Few studies have investigated the association between GR haplotypes and cortisol reactivity, and report consistent but small effects of GR SNPs in adults (for a review, see [10]). In infants, these associations have remained largely uncharted. GR haplotypes and FKBP5 were not related to attachment security or attachment disorganization. This is consistent with findings from previous studies, as main effects of candidate genes on attachment quality have not been reliably established [40]. Recently, Frigerio and colleagues [19] examined effects of attachment quality and candidate genes on alpha amylase and cortisol. They reported gene-environment interaction effects for alpha amylase, but no effects for cortisol. The current study investigated specific HPA-axis related genes in a large, population based sample, and provides evidence for effects of FKBP5, attachment quality and their interaction on cortisol reactivity.

The findings from the present study support the idea of interplay between genetic and environmental factors in explaining developmental outcomes [41]. Resistant attachment and FKBP5 predispose infants to increased cortisol reactivity both independently as well as in interaction. The current outcomes provide support for a double-risk model [42] as the combination of environmental (indexed by resistant attachment) and genetic (FKBP5) risks increase stress reactivity in an additive way, even in a rather homogeneous, low-risk sample. In a more diverse sample the gene-environment interaction effect might even be larger. Furthermore, it should be noted that careful assessment of the environment is essential for establishing GxE interactions. In the current study, the quality of the attachment relationship offers an observation-based but indirect index of the environment. Detailed direct observation of parenting quality in the natural setting may offer a more complete assessment, but was beyond the scope of the current study.

In sum, the present study shows HPA-axis related genes and attachment quality to be associated with stress reactivity both independently and in interaction. The combination of an insecure-resistant attachment relationship and carrying the minor allele of the FKBP5 gene is related to increased stress reactivity in infants.

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Chapter 4.2

Variation in the Glucocorticoid Receptor gene at rs41423247 moderates the effect of prenatal maternal psychological symptoms on child cortisol reactivity and behaviour.

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ABSTRACT

Background Prenatal maternal psychopathology affects child development, but some children seem more vulnerable than others. Genetic variance in hypothalamic-pituitary-adrenal-axis genes may influence the effect of prenatal maternal psychological symptoms on child emotional and behavioural problems. **Methods** This hypothesis was tested in the Generation R Study, a population-based cohort from fetal life onwards. In total, 1727 children of Northern European descent and their mothers participated in this study and were genotyped for variants in the Glucocorticoid Receptor gene (*GR*) (rs6189/rs6190, rs10052957, rs41423247, rs6195 and rs6198) and the FKBP5 gene (rs1360780). Prenatal maternal psychological symptoms were assessed at 20 weeks pregnancy and child behaviour was assessed by both parents at 3 years. In a subsample of 331 children data about cortisol reactivity was available. **Results** Based on power calculations, only those genetic variants with sufficient minor allele frequencies (rs41423247, rs10052957, rs1360780) were included in the interaction analyses. We found that variation in *GR* at rs41423247 moderates the effect of prenatal maternal psychological symptoms on child emotional and behavioural problems (beta 0.41, se 0.16, $p = 0.009$). This prenatal interaction effect was independent of mother's genotype and maternal postnatal psychopathology, and not found for prenatal psychological symptoms of the father. Moreover, the interaction between rs41423247 and prenatal psychological symptoms was also associated with decreased child cortisol reactivity (beta -2.30, *p*-value 0.05). **Conclusions** These findings emphasize the potential effect of prenatal gene-environment interaction, and give insight in possible mechanisms accounting for children's individual vulnerability to develop emotional and behavioural problems.

INTRODUCTION

Maternal psychopathology during pregnancy has been associated with a broad range of temperamental difficulties and emotional and behavioural problems in children, such as increased anxiety, poorer attention and hyperactivity [1], but the mechanisms accounting for these associations are only partly understood. It has been posited that in utero-exposure to stress increases fetal exposure to cortisol, which might influence the development of the fetal hypothalamic-pituitary-adrenal-axis (HPA-axis) [2-4]. Indeed, mounting evidence points to an effect of maternal depression, anxiety and stress during pregnancy on maternal cortisol levels, which in turn have been associated with increased cortisol levels and disrupted behaviour in the offspring [5-8]. Diathesis-stress models postulate that adversity in fetal life alters the development of neuronal and endocrine responses to stressors and predisposes individuals to disease [9].

Yet, not every child of mothers with psychological symptoms during pregnancy will actually develop emotional and behavioural problems [10]. This apparent individual vulnerability to the effect of maternal psychological symptoms may be partly explained by common variation in genes that regulate the HPA-axis. Prenatal gene-environment interaction is very plausible, but the empirical evidence is scarce [10]. The aim of the current study was to examine whether HPA-axis related genes moderate the association between prenatal maternal psychological symptoms and child emotional and behavioural problems.

The HPA-axis is the main neuro-endocrine system that is activated in response to stress. The axis consists of the hypothalamus, the anterior pituitary and the adrenal cortex. Glucocorticoids, i.e. cortisol, are the final effectors of the axis and exert a negative feedback effect on both cortico-tropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) production and secretion in the hypothalamus and the pituitary. In response to various physical and psychological stressors, the HPA-axis becomes activated and as a result the cortisol level increases. Cortisol exerts its effect via the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). In the brain, MR mediates the onset of the stress response, whereas GR is involved in the termination of the stress response [11]. Within the GR gene (*GR*, *NR3C1*) region, several single nucleotide polymorphisms (SNPs) (rs6189/rs6190, rs10052957, rs41423247, rs6195 and rs6198) have been studied in relation to HPA-axis function and psychiatric disorders. Associations have been found between *GR* SNPs and receptor sensitivity to cortisol (rs41423247, rs6195, rs6198, rs6189/rs6190), hippocampal and amygdala size (rs10052957), and psychiatric disorders, such as depression (rs41423247, rs6189/rs6190) and bipolar disorder (rs10052957) [12]. The sensitivity of the GR to cortisol is further influenced by the FK506 binding protein 5 (*FKBP5*), which acts as co-chaperone of *GR*. *FKBP5* SNPs (e.g. rs1360780) have been associated with insufficient recovery of cortisol

levels after psychosocial stress [13], with depression [14-15] and with the response to antidepressant treatment [16-17]. Hence, these SNPs are attractive candidates to moderate the effect of prenatal maternal psychopathology on child development.

We hypothesized that vulnerability to prenatal maternal psychological symptoms is accentuated by common variation in *GR* and *FKBP5*, which may result in an increased risk for emotional and behavioural problems. This hypothesis was tested in 1727 children participating in a large population-based cohort. First, we evaluated the moderating effect of child SNPs in *GR* and *FKBP5* on the association between prenatal maternal psychological symptoms and child emotional and behavioural problems, controlling for maternal genotype, postnatal maternal psychological symptoms and environmental factors. To study the plausibility of direct intra-uterine effects of maternal symptoms, we also evaluated the interaction between child SNPs and psychological symptoms of the father on child development. Second, in a subsample we examined whether child SNPs interact with prenatal maternal psychological symptoms to influence child cortisol reactivity.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from fetal life onwards in Rotterdam, the Netherlands. The Generation R Study has previously been described in detail [18-19]. All children were born between April 2002 and January 2006. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam. Written informed consent was obtained from all adult participants.

Population of analysis

In genetic analyses, population stratification can increase the rate of false positive findings in heterogeneous samples like the Generation R Cohort. Hence, we selected children of Northern European descent, which was determined by principle component analyses of genome wide association data, as described previously [20]. Principle component analyses yield factors that can be interpreted as the direction which maximizes the variance of the sample while being uncorrelated to previous components. Within the children of Northern European descent (n=2650), genetic data and information about prenatal maternal psychological symptoms were available in 2065 children. In 1727 (84%) of these children, data were available about child emotional and behavioural problems at the age of three years. These 1727 children comprised the population of

analysis. Data on cortisol reactivity was available in 331 children participating in a subsample of children followed in more detail; the Generation R Focus Study [20-21].

Genotyping

DNA was collected from cord blood at birth. Participants were genotyped for six HPA-axis related SNPs; rs6189/rs6190 (ER22/23EK), rs10052957 (TthIII1), rs41423247 (Bcl1), rs6198 (GR9beta), rs6195 (N363S) and rs1360780 [22]. These SNPs were chosen on the basis of their reported functionality [23]. Genotyping was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg, Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C, (15 minutes), then 40 cycles of 94° C (15 seconds) and 60° C (1 minute)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 97-99% of the samples. To confirm the accuracy of the genotyping 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1% for all genotypes. Contamination with maternal blood occurred in < 1% of cases. Mendelian errors occurred in < 0.5% of cases; these were excluded. Allele frequencies were in Hardy Weinberg Equilibrium (HWE) (p>0.05).

Maternal psychological symptoms

Maternal and paternal psychological symptoms were assessed at 20 weeks of pregnancy and two months postnatal with the Brief Symptom Inventory (BSI), a validated self-report questionnaire with 53 items to be answered on a five-point scale, ranging from "0 = not at all" to "4 = extremely" [24-25]. The Global Severity Index (GSI) is the sum score of all 53 items and defines a broad spectrum of psychological symptoms (Depression, Hostility, Anxiety, Phobic Anxiety, Psychoticism, Paranoid Ideation, Obsessive-Compulsive, Interpersonal Sensitivity, and Somatization). For prenatal psychological symptoms of the mother the Cronbach's alpha was 0.91; for fathers it was 0.93. For maternal postnatal psychological symptoms alpha was 0.93.

Child behaviour

The Child Behavior Checklist/1½-5 (CBCL/1½-5) was used to obtain standardized parent reports of children's emotional and behavioural problems at 3 years. This questionnaire contains 99 items, which are scored on a three-point scale: 0 = not true, 1 = somewhat true or sometimes true, and 2 = very true or often true, based on the two preceding months. The Total Problems score is obtained by summing the scores of all 99 items. Next to the Total Problems score, 6 syndrome scales are obtained; Emotionally Reactive, Anxious/Depressed, Somatic Complaints, and Withdrawn, Attention Problems and Ag-

gressive Behavior. The psychometric properties of the CBCL are well established [26]. The alpha for the CBCL Total problem scores as reported by the mother was 0.91; for the CBCL Total problem scores as reported by the father alpha was 0.93.

Child cortisol reactivity

Cortisol reactivity was assessed in response to stress evoked by the Strange Situation Procedure (SSP) [27] at age 14 months, in line with other studies [28-29]. The SSP measures the quality of the attachment relationship and is a widely used and well-validated procedure to evoke mild stress in infants. The procedure consists of seven episodes of 3 minutes each in which stress is evoked by the unfamiliar lab environment, a female stranger entering the room and engaging with the infant, and the parent leaving the room twice [19]. To assess cortisol reactivity, three saliva samples were taken using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany); the first prior to the SSP, the second directly after the SSP and the third 15 minutes later. Samples were centrifuged and frozen at -80°C. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 7% and 9%, respectively. For each time point, cortisol values above the 99th percentile (>200 nmol/L) were excluded (n = 12) from the analysis reducing the impact of outliers. For stress reactivity a delta was calculated between the last sample and the first sample. To control for the Law of Initial Values [30], which states that the direction of response of a body function depends to a large degree on the initial level, this delta was statistically adjusted for the first sample just prior to the stressful situation. This adjustment also controls for the different times of sampling.

Covariates

Gestational age was established by fetal ultrasound examinations. Information about Apgar score, birth weight and gender of the infant was obtained at birth. Information about maternal age, educational level, smoking during pregnancy, parity and child age was obtained by questionnaire. The inclusion of these potential confounders was primarily determined a priori and based on existing knowledge about the association between prenatal parental psychopathology and child development [1].

Statistical analysis

Differences in baseline characteristics of responders (n= 1727) and non-responders (n=334) to the CBCL/1½-5 were compared with the chi-square statistic for categorical variables, the independent t-test for normally distributed continuous variables and the

Mann Whitney U test for non-normally distributed continuous variables. The CBCL Total Problems scores of mother and father were square root transformed to achieve a normal distribution. Next, Total Problems scores and syndrome scores were z-standardized, summed and divided by two to obtain average scores based on both informants. Using the information of two raters strengthens the reliability of the outcome measure. Also, using a secondary informant reduced possible reporter bias because this information obtained of the father is less likely to be influenced of the prenatal report of the mother. If only the score of one parent was available, this score was used (12%). The syndrome scores could not be normalized and were analyzed as dichotomous variables. As Dutch norm scores have not been published, the 80th percentile was used as cut-off to differentiate between children with low and high scores on syndrome scales, in line with previous analyses [31]. Missing values on maternal SNPs, Apgar score and maternal psychological symptoms two months after child birth were imputed using a fully conditional specified model (max. 12% missings). Pooled estimates from the five imputed datasets were used to report the effect estimates (beta's) and their standard errors (se) [32]. Power analyses were performed using Quanto v1.2 [33]. The decision to include SNPs in the analysis was based on the criterion that the estimated power was 0.80 or above. To rule out gene-environment correlation, we computed correlations between child SNPs and prenatal maternal psychological symptoms (Spearman, two-tailed).

First, we tested for genetic and environmental main effects. To test our hypothesis, we examined whether child SNPs moderate the effect of prenatal maternal psychological symptoms on child emotional and behavioural problems using multivariate linear regression. This model assumes additive genetic interaction, and optimizes statistical power. In these analyses, prenatal and postnatal psychological problems of the parents were used as continuous variables. The final regression model testing the interaction between rs41423247 and prenatal maternal psychological symptoms included the following covariates: maternal SNPs, maternal age, maternal education, maternal smoking during pregnancy, parity, Apgar score 5 min. postnatal, child gender, gestational age, postnatal maternal psychological symptoms, and child age at time of assessment behaviour [32].

Second, to explore the specificity of these interactions, we tested whether child SNPs moderated the association between *postnatal maternal psychological symptoms* and child emotional and behavioural problems. We also tested whether child SNPs moderated the association between *prenatal psychological symptoms of the father* and child development. And we tested whether *maternal SNPs* also moderated the association between prenatal maternal psychological symptoms and child emotional and behavioural problems.

Third, we aimed to identify whether specific emotional and behavioural problems underlie the interactions and extended the findings to syndromes of emotional and

behavioural problems. In these logistic regression analyses multiplicative interaction is tested. For these analyses, we dichotomized the GSI on the 85th percentile to distinguish between mothers with and without prenatal psychological symptoms, in concordance with previous studies [34-35]. To study deviation from additive interaction between two risk factors, we calculated the synergy index (S). S equals [OR_{++}-1] / [(OR_{+-} -1) + (OR_{-+} -1)] and reflects the excess risk due to interaction relative to the risk from exposure to both determinants without interaction [36]. In the absence of interaction, S equals 1. Last, we explored the possible biological impact of the interaction between child SNPs and prenatal maternal psychological symptoms by focusing on a child cortisol reactivity. In these analyses, we used the dichotomized measure of prenatal maternal psychological symptoms, because in this subsample the residuals were no longer normally distributed if used as continuous measure.

Non-response analysis

Mothers who did not complete the CBCL/1½-5 (n=326) were on average younger (30.5 yrs. vs. 32.1 yrs., t = 5.94, p<0.001), were less likely highly educated (29.1% vs. 42.5%, $\chi 2$ =20.39(1df), p<0.001), and more likely to smoke during pregnancy (22.8% vs. 19.15%, $\chi 2$ =30.72(1df), p<0.001) than responding mothers (n=1727). Children of non-responding mothers had on average a lower birth weight (3478 gram versus 3583 gram, t=-3.50, p=0.001). The full characteristics of mothers and children in our study sample are presented in Table 1.

RESULTS

Table 2 presents the distribution of SNPs. Minor allele frequencies (MAFs) ranged from 3 to 37%. Power calculations left three SNPs for analyses; rs10052957 (MAF 31%), rs41423247 (MAF 37%) and rs1360780 (MAF 29%). Rs10052957, rs41423247 and rs1360780 were not significantly correlated with prenatal maternal psychological symptoms (rs10052957 Spearman's ρ = -0.016, p = 0.503; rs41423247 Spearman's ρ 0.009, p = 0.709; rs1360780 Spearman's ρ = -0.023, p = 0.345), which makes it less likely that gene-environment correlation was misinterpreted as interaction [37]. As presented in table 3, regression analyses indicated a significant main effect of prenatal maternal psychological symptoms on child emotional and behavioural problems (beta 1.23, p<0.001), which was slightly attenuated after adjustment for covariates and postnatal maternal psychological symptoms (beta 0.91, se 0.13, p <0.001). There were no genetic main effects.

Of the three candidate SNPS, rs41423247 moderated the association between prenatal maternal psychological symptoms and child emotional and behavioural problem scores. The interaction remained significant after adjustment for mother's genotype and

Table 1 Maternal and child characteristics (n=1727)

	Mean(sd) [a]
Mother	
Age at child birth	32.1 (3.8)
Educational level (%)	
Higher education	42.5
Smoking during pregnancy (%)	
Never	80.9
Parity	
First born	60.1
Child	
Gender (% boys)	50.4
Birth weight	3583.0 (506)
Gestational age (weeks)	40.4 (29.9;43.4) [b]
CBCL Total Problems score	18 (0;69) [b]

[a]unless otherwise indicated, [b]median(100%range). Abbreviations: CBCL; child behavior checklist

Table 2 Distribution of Single Nucleotide Polymorphisms located in the Glucocorticoid Receptor gene and the FKBP5 gene (n=1727).

SNPs	Chr.	Variant	Minor allele number of copies			MAF	HWE *p*-value
Glucocorticoid receptor gene (GR/NR3C1)			**0**	**1**	**2**		
rs6189/6190	5	GàA	1614	97	0	3	0.228
rs10052957	5	GàA	787	739	159	31	0.444
rs41423247	5	GàC	675	790	243	37	0.628
rs6195	5	AàC	1542	135	5	4	0.254
rs6198	5	AàG	1144	478	39	16	0.185
FKBP5 gene							
rs1360780	6	CàT	843	730	130	29	0.103

Abbreviations: SNPs, single nucleotide polymorphism; Chr., chromosome; MAF, minor allele frequency; HWE, Hardy Weinberg Equilibrium

potential confounders (model 3; beta 0.41, $p = 0.009$), and after correction for multiple testing ($p_{Bonferroni}$ 0.05/3=0.017).

This dose response effect of variation in *GR* at rs41423247 based on untransformed variables is displayed in figure 1 and shows that in children of mothers with prenatal psychological symptoms the risk of emotional and behavioural problems increases in heterozygous carriers and homozygous carriers of the minor allele (Cytosine (C)) at rs41423247. For instance, homozygous carriers of the minor allele (CC) with mothers that had prenatal psychological symptoms scored higher on emotional and behavioural problems (CBCL/1½-5 Total Problems score 41 than exposed non-carriers (GG) (CBCL/1½-5 Total Problems score 17. This moderation by rs41423247 was not observed

Table 3 Interaction effect of HPA-axis SNPs with prenatal maternal psychological symptoms on child CBCL Total Problems scores (sqrt) parent report at three years (n=1727)**.**

	CBCL/1½-5 Total Problems Score (sqrt) parent report at 3yrs.								
	model 1 unadjusted			**model 2** adjusted for maternal genotype			**model 3** additionally adjusted for confounders[a]		
	beta	**se**	***p*-value**	**beta**	**se**	***p*-value**	**beta**	**se**	***p*-value**
Prenatal maternal psychological		1.23	0.11	<0.001 symptoms (E)					
rs10052957	0.04	0.03	0.272						
rs10052957 x E	0.22	0.18	0.204	0.23	0.18	0.200	0.22	0.17	0.200
rs41423247	0.04	0.03	0.210						
rs41423247 x E	0.38	0.16	0.018	0.38	0.16	0.018	0.41	0.16	0.009
rs1360780	-0.01	0.03	0.710						
rs1360780 x E	0.31	0.18	0.078	0.31	0.18	0.079	0.27	0.18	0.124

[a]model 3; adjusted for maternal genotype, maternal age, maternal education, maternal smoking during pregnancy, parity, Apgar score 5 min. postnatal, child gender, gestational age, postnatal maternal stress, and child age at time of assessment behaviour.

in children with mothers that had low prenatal psychological symptoms (CC CBCL/1½-5 Total Problems score 17 vs. GG CBCL/1½-5 Total Problems score 18). The figure based on the square root transformed outcome measure is presented in the supplementary material (figure S1).

Further evaluation of this prenatal GxE indicated that child rs41423247 neither interacted with *postnatal* maternal psychological symptoms (n=1501, beta 0.07, $p = 0.64$) nor with prenatal psychological symptoms of *the father* (n=1463, beta -0.15, $p = 0.53$) to influence the risk of child emotional and behavioural problems. Furthermore, *maternal genotype* at rs41423247 did not interact with prenatal psychological symptoms (n=1488, beta 0.15, $p = 0.36$).

We extended this finding to syndromes of child problems. These analyses revealed that the interaction between rs41423247 and prenatal maternal psychological symptoms significantly increased the risk for aggressive behaviour (aOR 1.76, 95%CI 1.12; 2.74, p=0.014) and anxious/depressed behaviour (aOR 1.71, 95%CI 1.07; 2.73, p =0.025) (Table S1). The synergy indices for these findings were 3.73 for the Aggression syndrome and 2.94 for the Anxious/Depressed syndrome indicating deviation from additivity.

To evaluate the biological impact, we tested for the interaction between rs41423247 and prenatal maternal psychological symptoms on cortisol reactivity in 331 toddlers. We did not find main effects of rs41423247 (beta -0.13, se 0.84, $p = 0.882$) and prenatal maternal psychological symptoms (beta -0.14, se 0.42, $p = 0.743$) on cortisol reactivity. Moreover, rs41423247 interacted with prenatal maternal psychological symptoms to influence child cortisol reactivity after stress (beta -2.30, se 118, p-value 0.053) (Figure 2).

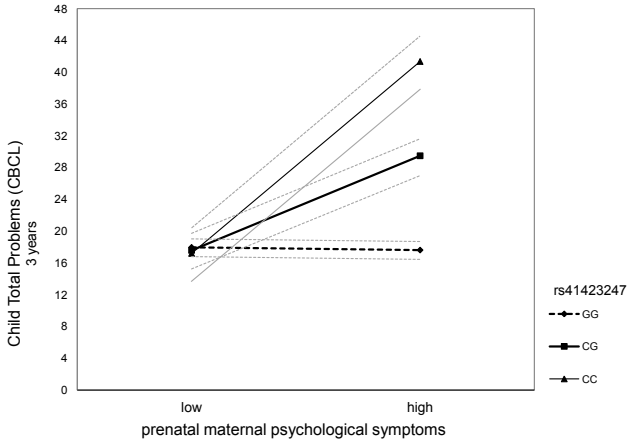


Figure 1 Results of adjusted linear regression estimating the association between low and high prenatal maternal psychological symptoms on child emotional and behavioral problems at age 3 as a function of variance in *GR* at rs41423247 (n=1704). The main effect of variance at rs41423247 was not significant (b = 0.04, p = 0.210) . The main effect of prenatal maternal psychological symptoms was significant (b = 1.23, $p < 0.001$) and the G x E interaction was in the expected direction (b = 0.38, p = 0.018). The interaction showed a dose response effect of the minor allele at rs41423247 on the risk of emotional and behavioral problems in children of mothers with high scores on prenatal maternal psychological symptoms, which was absent in children of mothers reporting low on prenatal maternal psychological symptoms. The dotted lines represent the 95% confidence intervals around the effect estimates.

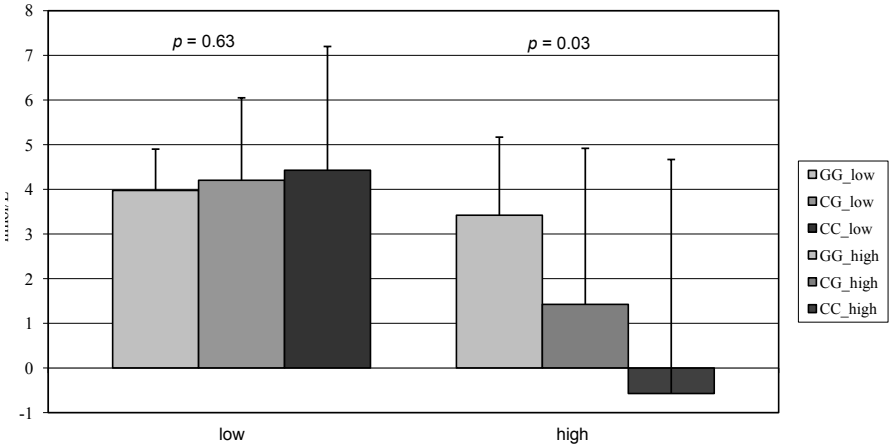


Figure 2 Results of linear regression analyses estimating the association between low (<85th%ile)and high (≥85th%ile) prenatal maternal psychological symptoms and child cortisol reactivity after stress as a function of variance in *GR* at rs41423247. The main effect of variance at rs41423247 was not significant (b = -0.13, se = 0.84, p = 0.882) . The main effect of prenatal maternal psychological symptoms was not significant (b = -0.14, se = 0.42, p = 0.743). The G x E interaction was in the expected direction (b = -2.30, se = 1.18, p = 0.05). The minor allele at rs41423247 was associated with decreased cortisol reactivity in children of mothers with high prenatal maternal psychological symptoms (beta -2.00, se 0.87, p = 0.03), which was absent in children of mothers reporting low prenatal maternal psychological symptoms (beta 0.23, se 0.47, p = 0.63).

If the mother reported high prenatal maternal psychological symptoms, children with the CC genotype showed significantly less cortisol reactivity than children with the GG genotype (beta -2.00, se 0.87, $p = 0.026$). Post hoc analyses revealed that child cortisol levels 15 minutes after the SSP accounted for the prenatal interaction effect on cortisol reactivity (beta -2.67, se 1.32, $p = 0.044$) (Table S2). The effect of the minor allele on cortisol reactivity was not found in children of mothers with low prenatal maternal psychological symptoms (beta 0.23, se 0.47, $p = 0.629$).

DISCUSSION

The current study investigated children's individual vulnerability to maternal psychological symptoms during pregnancy and the effect of this prenatal GxE on emotional and behavioural problems later in life. We found that a common variant in *GR* at rs41423247 (BclI) moderates the relation between prenatal maternal psychological symptoms and child emotional and behavioural problems. Maternal genotype at rs41423247 and postnatal maternal symptoms did not account for this effect. Also, the interaction between rs41423247 and prenatal symptoms of the father was not significant. Together, these findings seem to provide evidence for a prenatal GxE of child rs41423247 and prenatal maternal psychological symptoms, which influences the intra-uterine environment and results in a increased risk for emotional and behavioural problems in preschool children. Moreover, this prenatal gene-environment interaction may also affect HPA-axis function, as we found attenuated cortisol reactivity in response to stress in a subsample of 14-months-old carriers of the minor allele at rs41423247, but only if they had mothers with prenatal maternal psychological symptoms.

The single nucleotide polymorphism at rs41423247 is located in intron B, 647 bp downstream of exon 2, and results in a guanine to cytosine alteration [22]. The direction of the results of our study conform to previous research reporting an association between rs41423247 and increased sensitivity to glucocorticoids following a DEX-CRH test, lower cortisol response after psychological stress [12, 22], and major depression disorder [38-39]. The mechanism by which this SNP exerts its effect remains unclear since it is not located in a coding, regulatory or splicing part of *GR*. Recently, it was shown that the minor allele is associated with decreased abundance of the predominant GRalpha receptor isoform in the dorsolateral prefrontal cortex, which results in abnormal GR expression [40].

Katz and colleagues found increased expression of NR3C1 and FKBP5 during pregnancy. In depressed women, the increase of FKBP5 expression was smaller than in non-depressed women. There was no difference in NR3C1 expression between depressed and non-depressed women [41]. Also, rs41423247 is located in a single linkage

disequilibrium block with several other SNPs [42]. Hence, rs41423247 could also be a marker of the actual functional variant, possibly located in the promoter region of *GR*. In the current study, information about methylation status was not available. In rats, DNA methylation of the *GR* promoter region, which influence GR expression [43], has been associated with the programming effect of maternal licking and grooming on the HPA-axis [44]. Importantly, programming of the HPA-axis in rat occurs during the early postnatal period, whereas in humans neuroendocrine development takes place before birth [4]. This could explain our finding that rs41423247 specifically moderates the effect of maternal psychological symptoms during pregnancy, and not after birth.

Maternal depressed mood during pregnancy has been associated with increased methylation of the *GR* promoter region in neonates, which is turn predicted increased HPA-axis reactivity in these neonates at 3 months [45]. In contrast, chronic stress, like depression, is typically related to flattening of daytime cortisol rhythms and a blunted cortisol response, which has been described in children raised in neglectful environments [46]. In the general population, parental depression has been related to attenuated cortisol reactivity in response to stress in adolescents [47]. We showed that also in a population-based cohort of relatively healthy young children, the interaction between rs41423247 and prenatal maternal psychological symptoms resulted in an attenuated cortisol response. Since cortisol reactivity at the age of 14 months and problem behaviour at 3 years were not related we should be careful to infer a causal pathway. Hence we can only speculate whether lower cortisol reactivity is more a global indication of HPA-axis vulnerability or part of the underlying pathophysiology of child problems. Importantly, it also indicates a consistency in results and makes a chance finding less likely.

There was no significant main effect of rs41423247 on the risk of child emotional and behavioural problems. Replicable genetic main effects are seldom found in psychiatric genetics [48]. This observation from linkage analyses and candidate gene studies is underscored by genome wide association studies (GWAS). Although expectations were high, the genome wide approach has not yet found the genes accounting for the high heritability estimates of most psychiatric disorders obtained in twin studies. In search for this missing heritability, it has been posited that at least part of it must be hidden in gene-environment interactions [49]. Gene-environment interaction are not conditional on the presence of main effects. If the genetic effect is only apparent in the high range of the environmental stressor and not in the low range, there may indeed be gene-environment interaction without a genetic main effect [48, 50].

Few studies reported GxE with *GR* SNPs. Bet and colleagues reported an interaction of rs6189/rs6190 and rs6198 with childhood adversity on adult depression [51]. Interactions have been reported for *FKBP5* SNPs with childhood abuse and risk for PTSD [52], and with infant attachment on cortisol reactivity in children [19]. To the best of our knowledge this is the first study to report moderation by rs41423247.

There are several limitations to this study. First, even in this large population-based cohort we did not have sufficient power to study all a priori selected candidate SNPs. So, we restricted the analyses to those SNPs with a MAF for which the present study yielded sufficient power. Second, observational measurements in this large cohort were not feasible. Therefore, we relied on report of parents on psychological problems and child behaviour. Yet, we used validated questionnaires with good reliability and validity. Third, to reduce possible bias due to population heterogeneity only children of Northern European descent were included in the analyses. Therefore, we should be careful generalizing our findings to other populations. Fourth, information about child cortisol levels was only available in a subsample. Currently, saliva samples are being collected in the children at the age of five. In the future, these samples will provide us with information about cortisol daily rhythms in a much larger sample. This will enable us to further study the mechanisms underlying the interaction of child allelic variants with prenatal maternal psychological symptoms and HPA-axis regulation.

In conclusion, we found evidence for prenatal gene-environment interaction of *GR* rs41423247 with maternal psychological symptoms resulting in an increased risk of child emotional and behavioural problems. This interaction also seems to affect child cortisol reactivity. These findings emphasize the potential effect of prenatal programming on child development, and give further insight in possible mechanisms accounting for the differences in children's vulnerability to maternal psychological symptoms.

SUPPLEMENTARY MATERIAL

Table S1 Interaction effect of the minor allele at rs41423247 and prenatal maternal psychological symptoms on subtypes of child emotional and behavioural problems reported by both parents (n=1704).

	CBCL/1½-5 Syndrome scales, parent report at 3yrs.											
	Emotionally Reactive		Anxious/ Depressed		Somatic Complaints		Withdrawn		Attention Problems		Aggression	
Child SNP* prenatal maternal psychological symptoms (E)	**aOR** [a]	**95%CI**	**aOR**	**95%CI**	**aOR**	**95%CI**	**aOR**	**95%CI**	**aOR**	**95%CI**	**aOR**	**95%CI**
rs41423247 x E	1.17	0.76;1.80	1.71**	1.07;2.73	1.10	0.70;1.72	1.24	0.80;1.92	1.51*	0.96;2.38	1.76**	01.12;2.74

[a] adjusted ORs represent the increased risk of emotional and behavioral problems per unit increase of the determinants, adjusted for maternal genotype, maternal age, maternal education, maternal smoking during pregnancy, parity, Apgar score 5 min. postnatal, child gender, gestational age, postnatal maternal psychological symptoms, and child age at time of assessment behaviour.

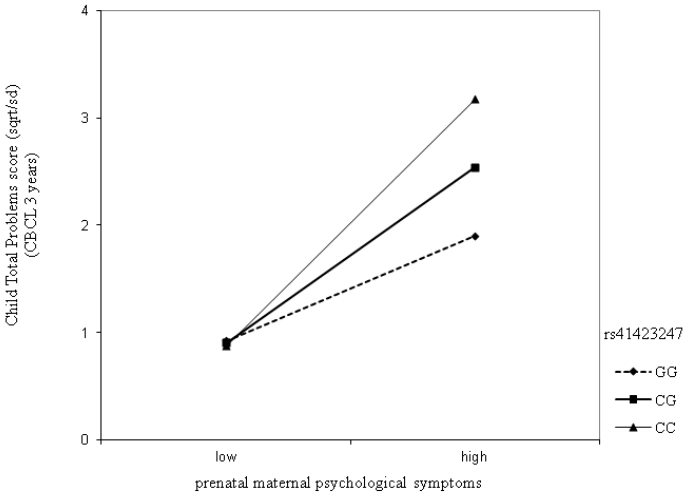
Abbreviations: SNP; single nucleotide polymorphism, aOR; adjusted odds ratio, 95%CI; 95% confidence interval.

p-values *<0.10, ** <0.05

Table S2 Interaction effect of variance at rs41423247 with prenatal maternal psychological symptoms on child cortisol reactivity at 14 months (n=331).

	Child cortisol (nmol/l) at 14 months											
	Baseline cortisol levels before the SSP[b]			Cortisol levels directly after the SSP[b]			Cortisol levels 15 min. after the SSP[b]			Cortisol reactivity[a]		
	beta	se	*p*	beta	se	*p*	beta	se	*p*	beta	se	*p*
rs41423247 x prenatal maternal psychological symptoms	0.19	1.19	0.88	-0.75	1.08	0.49	-2.68	1.32	0.04	-2.30	1.18	0.05

[a] cortisol reactivity = cortisol levels 15 minutes after the SSP – baseline cortisol levels, adjusted for baseline cortisol levels
[b] SSP; strange situation procedure



SM Figure 1 Results of adjusted linear regression estimating the association between low and high prenatal maternal psychological symptoms on child emotional and behavioral problems at age 3 as a function of variance in *GR* at rs41423247 (n=1704). The main effect of variance at rs41423247 was not significant ($b = 0.04$, $p = 0.210$) . The main effect of prenatal maternal psychological symptoms was significant ($b = 1.23$, $p < 0.001$) and the G x E interaction was in the expected direction ($b = 0.38$, $p = 0.018$). The interaction showed a dose response effect of the minor allele at rs41423247 on the risk of emotional and behavioral problems in children of mothers with high scores on prenatal maternal psychological symptoms, which was absent in children of mothers reporting low on prenatal maternal psychological symptoms. The dotted lines represent the 95% confidence intervals around the effect estimates.

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Chapter 5

Gene-environment interaction; the Serotonin Transporter gene

Chapter 5.1

Serotonin Transporter Polymorphism Moderates Effects of Prenatal Maternal Anxiety on Infant Negative Emotionality

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ABSTRACT

Background Consistent with the fetal programming hypothesis effects of maternal prenatal anxiety have been found to predict various measures of infant temperament in the early postnatal period. In recent years, a polymorphism in the serotonin transporter gene (5-HTTLPR) emerged as a moderator of diverse environmental influences on different outcomes with individuals carrying the short allele being generally more vulnerable to adversity. **Methods** We tested whether the association between self-reported maternal anxiety at 20 weeks gestation (Brief Symptom Inventory) and mother rated infant negative emotionality at six months postpartum (Infant Behavior Questionnaire-Revised) would be moderated by the 5-HTTLPR in a large Dutch cohort sample (N = 1513). We hypothesized that infants carrying the 5-HTTLPR short allele would be more susceptible and therefore more affected by both low and high prenatal maternal anxiety vis-à-vis negative emotionality than other genotypes. **Results** Findings of a significant GXE interaction (B = .65, p = .01) were supportive of a vulnerability model with infants carrying the short allele being more negatively emotional when mothers reported anxiety during pregnancy while there was no difference between genotypes on negative emotionality when maternal anxiety was low. **Conclusions** The association between maternal anxiety during pregnancy and negative emotionality in early infancy was significant in infants carrying one or more copies of the short allele, but not in those homozygous for the long allele. The 5-HTTLPR short allele may increase vulnerability to adverse environmental influences as early as the fetal period.

INTRODUCTION

Early experiences and environmental influences have been found to shape human development as early as the fetal period. This observation has been interpreted in terms of the fetal programming hypothesis (1, 2) which stipulates that the fetus adjusts its phenotype (e.g., metabolism and stress reactivity) in utero––on the basis of placental transferred maternal nutritional and hormonal cues about the "outside" world––as a means of optimally adapting to the (anticipated) conditions of the postnatal environment.

Findings consistent with the fetal programming hypothesis have been reported repeatedly (3), along with perhaps related evidence linking prenatal maternal anxiety and mother-reported infant temperament. For example, higher levels of maternal anxiety during pregnancy has been found to predict (a) greater infant temperament reactivity at eight-weeks postpartum (4); (b) greater infant negative behavioral reactivity at four-months postpartum (5); (c) greater infant difficult temperament at four- and six-months postpartum (6); and (d) decreased infant attention regulation at three- and eight-months postpartum (7). The fact that all of the just-cited investigations controlled for postnatal maternal anxiety clearly suggests that the predicted differences in infant temperament are a function of prenatal maternal anxiety rather than just of postnatal maternal psychological state.

What the available research has yet to address is whether the putative effects of maternal anxiety on infant temperament vary across fetuses due to their genetic make-up. That this might be the case is certainly suggested by recent studies of gene-X-environment (GXE) interaction. Most prominently, perhaps, a polymorphism in the serotonin transporter promoter gene area (SLC6A4), the 5-HTTLPR, has been found to moderate the apparent effect of adverse early environmental influences on a variety of phenotypic outcomes. For example, severe childhood maltreatment has been associated with more depression symptoms in adulthood in individuals that carried one or two copies of the short 5-HTTLPR allele but not in individuals homozygous for the long allele (8). Similarly, low maternal sensitivity at seven months predicted insecure attachment at 15 months exclusively for infants carrying 5-HTTLPR short alleles, whereas attachment quality of infants homozygous for the long allele was independent of observed levels of maternal sensitivity (9).

Most such GXE results have been interpreted in terms of diathesis-stress thinking (10), with the 5-HTTLPR short allele regarded as a vulnerability factor (or diathesis) predisposing individuals toward problematic functioning (e.g., depression) in the face of contextual adversity (e.g., child maltreatment). But as noted by Taylor et al. (11) in their study of a GXE interaction involving 5-HTTLPR and quality of the early family environment in the prediction of adult depression, as well as by Belsky and associates (12,

13) in their analysis of many other GXE findings, the short allele may perhaps be better conceptualised as a "plasticity gene" rather than a "vulnerability gene." This is because individuals with the short allele appear in some research to be not only more likely than others to succumb to the negative effects of adverse environments, but also more likely than others to benefit from positive supportive ones (14). This proves true even in work in which environmental support is operationalized as merely the absence of negative contextual conditions (e.g., no childhood maltreatment).

Evidence of this kind is consistent with Belsky's (13, 15-17) *differential-susceptibility hypothesis* which posits that some individuals, including those with the short allele of the 5-HTTLPR, are more affected by both negative *and* positive environmental conditions than are others (i.e., for better and for worse)—rather than just disproportionately and negatively affected by contextual adversity than others (see also 11, 18). A recent reanalysis of data from Neuman and associates' (19) GXE study of effects of maternal smoking during pregnancy on ADHD in childhood (20) provided first evidence of genetically-related differential susceptibility to effects of prenatal experiences. The study, however, investigated the moderating effect of DRD4 and not 5-HTTLPR. Children carrying the dopamine DRD4 7-repeat allele––an allele repeatedly associated with differential susceptibility (12, 13, 21)––tended to be most *and* least likely to develop ADHD, depending, respectively, on whether their mothers did or did not smoke during pregnancy. It remains to be determined whether the effect of stressful prenatal experiences is moderated in a manner consistent with differential susceptibility when the moderator is 5-HTTPLR.

In light of evidence that (a) prenatal maternal anxiety predicts infant temperament and (b) that the short allele of 5-HTTPLR may function as a plasticity gene, moderating environmental influences in a for-better-and-for-worse manner (17), the current study tested whether the temperaments of infants with one or two short alleles would be more affected by maternal prenatal anxiety than those homozygous for long alleles, and whether this moderation would be more consistent with a differential susceptibility than diathesis-stress model. That is, whether they would prove to be less negatively emotional than others under conditions of low maternal prenatal anxiety, yet more negatively emotional than others under conditions of high maternal prenatal anxiety.

METHODS AND MATERIALS

Design

This research was embedded in the Generation R Study, a population-based cohort study investigating growth, development and health from fetal life into young adulthood in Rotterdam, the Netherlands. The Generation R Study has previously been described

in detail (22). Briefly, all pregnant women living in the study area with a delivery date between April, 2002 and January, 2006 were informed about the research project by community midwives and obstetricians. Inclusion criteria were: (1) residency in study area at delivery data; (2) delivery date between April 2002 and January 2006; and (3) informed consent. Importantly, mothers with psychiatric disorders were not identified nor excluded from study participation. Written informed consent and genetic data was available for 4345 study families. The Generation R study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam (numbers: prenatal, MEC 198.782/2001/31 and postnatal, MEC 217.595/2002/202).

Participants

Only infants with at least one parent of self-reported Dutch ethnicity were included in the present study in order to avoid confounding effects of ethnic differences in gene frequency. Of the 3,639 qualifying Dutch families 1,513 had data on 5-HTTLPR, infant negative emotionality, and prenatal maternal anxiety and were consequently included in the study; see Table 1 for sample characteristics. (When analyses were restricted to the 1,136 infants with two parents of Dutch ethnicity, results remained the same.) Comparisons between included and excluded families revealed no significant differences regarding 5-HTTLPR, infant negative emotionality, infant gender, and postnatal maternal anxiety or depression. Significant differences emerged, however, for prenatal maternal anxiety which was significantly greater for excluded than included mothers (M = .22, SD = .38 versus M =.18, SD = .31, p <.01, d = .14), and for some variables which are not reported here given restricted space (and are available on request).

Maternal prenatal and postnatal psychopathology

Maternal psychopathology was assessed at 20 weeks of pregnancy and at six-months postpartum with the Brief Symptom Inventory (BSI), a validated self-report questionnaire with 53 items answered on a five-point scale ranging from 0 = "not at all" to 4 = "extremely" (23-25). The BSI is a short version of the Symptom Checklist 90 (SLC-90) (26) and defines a broad spectrum of psychiatric symptoms over the preceding seven days. For this study, the prenatal and postnatal anxiety and the postnatal depression subscales were used.

Infant negative emotionality

Infant temperament was assessed at six months postpartum using an abbreviated version of the Infant Behavior Questionnaire – Revised (IBQ-R) (27). This measure is based on maternal reports of frequencies of specific infant behaviors observed over the past week. Only six of the original 14 IBQ-R subscales were administered and the original seven-point response scale was truncated to a three-point scale (0 = never present; 1

Table 1 Demographic Characteristics of the Sample (n = 1513)

Variables	N (%)
Age at first contact (years)	*M* = 31.81, *SD* = 4.03 (Range: 17-43)
Educational Level	
No education	20 (1.3%)
Low (12 years or less)	129 (8.5%)
Mid-low (13-15 years)	369 (24.4%)
Mid-high (16-17 years)	399 (26.4%)
High (18 years or more)	596 (39.4%)
Living Situation	
Living with partner	1443 (95.3%)
Living without partner	70 (4.6%)
Income	
< 1200 Euro	69 (4.6%)
1200-2200 Euro	261 (17.3%)
> 2200 Euro	1183 (78.2%)
Smoking during pregnancy	174 (11.5%)
Alcohol during pregnancy	861 (56.9%)
Anxiety during pregnancy	*M* = .18, *SD* = .31
Anxiety at 6 months postnatal	*M* = .22, *SD* = .36
Depression at 6 months postnatal	*M* = .16, *SD* = .35
Child Gender	
Boy	761 (50.3%)
Girls	752 (49.7%)
Child gestational age at birth (weeks)	*M* = 40.16, *SD* = 1.44
Child birth weight (grams)	*M* = 3552.40, *SD* = 508.28
Child 5-HTTLPR	
l/l	497 (32.8%)
s/l	738 (48.8%)
s/s	278 (18.4%)
Child negative emotionality at 6 months	
Fear	*M* = 0.33, *SD* = 0.27
Distress to Limitations	*M* = 0.62, *SD* = 0.30
Recovery of Distress	*M* = 1.56, *SD* = 0.28
Negative Emotionality Composite (standardized)	*M* = .00, *SD* = 2.18

= sometimes present; 2 = often present) after a pilot study revealed that respondents seldom used the extreme positions of scales (28). Total scores for each subscale were calculated by dividing the sum of the items by the number of endorsed items. Internal consistencies for the adapted IBQ-R ranged from .71 to .85––similar to the internal consistencies of the original IBQ-R (27). A composite measure for infant negative emotionality was derived by standardizing and averaging three of the subscales (distress to

limitations, fear, and recovery of distress [reflected]). Higher scores on this composite measure represent greater negativity.

Covariates

Birth weight and infant gender were obtained from midwife and hospital registries shortly after birth. Gestational age was established by fetal ultrasound examinations. Information about income, maternal educational level, maternal smoking and maternal alcohol consumption during pregnancy was obtained by questionnaires. The highest completed education determined the educational level of the mothers. Following the definition of Statistics Netherlands (29), educational level was categorized as *no education*, *low* (12 years of education or less), *mid-low* (13-15 years), *mid-high* (16-17 years), and *high* (18 years or more). Maternal smoking and maternal alcohol consumption were assessed in the first, second and third trimester and summarized as either "yes, at least sometime during pregnancy" or "never during pregnancy".

Genotyping

DNA was derived from cord blood samples at birth. The 43-base pair insertion/deletion in the promoter region of the 5HTT gene was genotyped using Taqman allelic discrimination. Primer sequences were taken from Hu et al. (30). Reactions were performed in a 384-wells format in a total volume of 5 ul containing 2 ng DNA, 120 nM FAM-probe, 80 nM VIC-probe, PCR primers (100 nM each), dimethyl sulfoxide (DMSO) (4% by volume), and 1 x genotyping master mix (Applied Biosystems Inc.). PCR cycling consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 96° C and annealing and extension for 90 seconds at 62.5° C. Signals were read with the Taqman 7900HT (Applied Biosystems Inc.) and analyzed using the sequence detection system 2.3 software (Applied Biosystems Inc.). To evaluate genotyping accuracy, 225 random samples were genotyped a second time. No discrepancies were found. To check for potential contamination with maternal blood, gender was determined in male participants. Contamination occurred in < 1% of cases, which were excluded. Genotype distribution (l/l: 32.8%; l/s: 48.8%; s/s: 18.4%) conformed to the Hardy-Weinberg Equilibrium ($p > .99$).

Statistical Analysis

Unadjusted associations between the different measures were evaluated using bivariate correlations (Pearson, two-tailed). The moderating effect of 5-HTTLPR was tested with a hierarchical regression model. All variables included in the regression analysis were centered. Missing data occurred in this longitudinal project due to attrition and failure to complete all assessments, as follows: maternal education (1.0%), living with partner (2.7%), income (5.5%), six-month maternal depression (0.1%), drinking during pregnancy

(4.1%), IBQ-R fear (0.6%), IBQ-R distress to limitations (2.0%), and IBQ-R recovery from distress (3.6%). Missing data were imputed using multiple imputation (31). Test statistics and regression coefficients were averaged across five imputed data sets. When analyses were run using only cases with complete data, results did not differ from those derived from the imputed data sets. The level of significance for all analyses was set at $\alpha = .05$.

Given the negative findings of a recent meta-analysis of GXE studies involving 5-HTTLPR and life event stress in the prediction of adult depression (32), the robustness of any GXE interaction discerned in the research reported herein is rigorously evaluated by randomly dividing the sample into two subsamples and determining if the results which emerge from each can be cross-validated on the other, following procedures pioneered by Bakermans-Kranenburg, Van IJzendoorn and Kroonenberg (33). All statistical analyses were carried out using PASW Statistics, version 18.0 for Windows (34).

RESULTS

According to bivariate correlations, 5-HTTLPR was not associated with infant negative emotionality and, critically, maternal prenatal anxiety. The latter fact rules out the possibility of gene-environment correlation being misinterpreted as GXE interaction (17). See Table 2 for the bivariate correlations between variables.

For the hierarchical regression analysis, variables were entered in three steps to predict infant negative emotionality: step 1 included all the covariates, step 2 infant 5-HTTLPR (0, 1, 2 for, respectively, l/l, s/l, and s/s) and maternal anxiety during pregnancy, and step 3 the 2-way interaction between 5-HTTLPR and maternal prenatal anxiety. Income, depression at six-months postpartum and anxiety during pregnancy significantly predicted infant negative emotionality. Most importantly, although there was no main effect of 5-HTTLPR, the interaction between 5-HTTLPR and maternal prenatal anxiety was significant ($B = .65$, $p = .01$, Effect Size (f^2) = .004) in the prediction of infant negative emotionality six-months after birth (see Table 3). Running the regression model separately for males ($n = 761$) and females ($n = 752$) did not reveal any sex differences, though the interaction term for males was only marginally significant ($B = .55$, $p = .09$) while it remained significant for females ($B = .87$, $p = .03$). In order to investigate the small effect size of the interaction term for the full sample ($f^2 = .004$) we ran additional hierarchical regression models––stratified by genotype––with step 1 including the same covariates as above and step 2 maternal anxiety during pregnancy. Although there was no significant effect of maternal prenatal anxiety on negative emotionality for infants homozygous for the long allele ($B = .27$, $p = .43$, $f^2 = .001$), significant effects emerged for heterozygous infants ($B = .63$, $p = .05$, $f^2 = .005$) and for infants homozygous for the short

Table 2 Unadjusted Associations between Variables (N = 1513)

	Variables	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Maternal Age	––												
2	Maternal Education	**.32****	––											
3	Living with Partner (1 = yes ; 2 = no)	**-.15****	**-.14****	––										
4	Income	**.28****	**.39****	**-.39****	––									
5	Smoking during pregnancy (1 = no; 2 = yes)	**-.06***	**-.27****	**.10****	**-.21****	––								
6	Alcohol during pregnancy (1 = no; 2 = yes)	**.22****	**.26****	<.01	**.14****	**.07***	––							
7	Anxiety during pregnancy	**-.12****	**-.13****	**.15****	**-.21****	**.16****	-.02	––						
8	Anxiety at 6 months postnatal	**-.06***	**-.06***	**.11****	**-.11****	**.10****	-.01	**.47****	––					
9	Depression at 6 months postnatal	**-.10****	**-.09****	**.11****	**-.17****	**.15****	<.01	**.42****	**.70****	––				
10	Child Gender (1 = male ; 2 = female)	.02	.04	-.01	**.08****	-.04	-.01	-.03	-.04	**-.06***	––			
11	Child Gestational Age at Birth	.01	**.08****	-.01	.01	.01	**.07****	.01	.04	.03	**-.05***	––		
12	Child Birth Weight	**.09****	**.10****	**-.07***	**.07****	**-.12****	.05	-.03	.05	.03	**-.12****	**.51****	––	
13	Child 5-HTTLPR (0 = l/l; 1 = s/l; 2 = s/s)	-.03	<.01	<.01	<.01	<.01	.01	-.01	.02	**.05***	.01	<.01	-.02	––
14	Child Negative Emotionality at 6 months	**-.08****	**-.06***	**.12****	**-.16****	.01	**-.06***	**.16****	**.16****	**.17****	<.01	.04	.01	<.01

Note. * $p < .05$. ** $p < .01$.

allele ($B = 1.39$, $p < .01$, $f^2 = .033$). Thus, for infants carrying one or more short alleles, greater prenatal anxiety predicted more negative emotionality.

The sample was randomly split into two subsamples of 767 and 747 cases and the robustness of the regression model was re-tested by cross-validation of the regression equation in each subsample. The regression equation for subsample 1 ($R = .28$) showed a cross-validation correlation for subsample 2 of .25 and the equation for subsample 2 ($R = .28$) showed a cross-validation correlation for subsample 1 of .25. To investigate the sensitivity of the predicted scores with respect to the exact form of the regression equation, the estimated scores for infant negative emotionality from both regression equations were also correlated within each subsample. The correlation between the two estimates within subsample 1 was $r = .93$ and within subsample 2 $r = .94$. Thus, the predicted scores from both regression models appeared to be largely similar within the two subsamples, thereby indicating that the equation coefficients of the regression model in this study were highly robust.

Table 3 Summary of hierarchical regression analysis

	Predictor Variables	Infant Negative Emotionality at 6 months B
Step 1		
	Maternal Age	-.01
	Maternal Education	.04
	Living with Partner (1 = yes; 2 = no)	.50
	Income	**-.47****
	Smoking during pregnancy (1 = no; 2 = yes)	-.28
	Alcohol during pregnancy (1 = no; 2 = yes)	-.19
	Anxiety at 6 months postnatal	.28
	Depression at 6 months postnatal	**.53***
	Child gestational age at birth	.06
	Child gender (1 = male; 2 = female)	.08
	Child birth weight	<.01
Step 2		
	5-HTTLPR	-.01
	Anxiety during pregnancy	**.62****
Step 3		
	5-HTTLPR x Anxiety during pregnancy	**.65****

Note. The displayed coefficients of the variables at steps 1 and 2 represent the values after inclusion of interaction terms at step3; N = *1513*, after Step 3: adjusted R^2 = .06**, $F_{(14,1498)}$ = 7.74, p <.01. * p < .05. ** p < .01.

In order to illuminate the nature of the interaction, we plotted regression slopes of maternal anxiety during pregnancy vis-à-vis infant negative emotionality separately for each of the three genotypes (see Figure 1). These simple slopes revealed what Belsky and Pluess (13) labeled a "plasticity gradient": The positive relation between prenatal maternal anxiety and postnatal infant negative emotionality being strongest for infants homozygous for the short allele (β = .28, p < .01), intermediate for heterozygotes (β = .18, p < .01), and weakest (and only marginally significant) for those homozygous for the long allele (β = .08, p = .06). After z-transformation of the standardized regression coefficients (35), the slopes of infants with s/s and s/l genotype were significantly larger than that of l/l genotypes (p < .01 and p < .05, respectively), whereas the difference of slopes between s/s and s/l was only marginally significant (p = .08).

Consideration of Figure 1 indicates that the results are more consistent with a diathesis-stress/dual-risk than a differential-susceptibility model of environmental action, because infants with at least one short allele had the highest negative emotionality scores when mothers reported prenatal anxiety but did not differ from those homozygous for

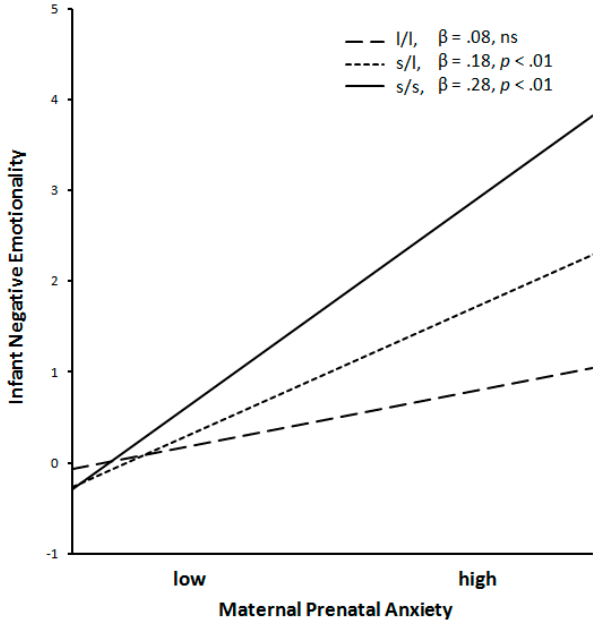

Figure 1 Linear relations between maternal reports of anxiety during pregnancy and infant emotional negativity at six months postpartum as a function of 5-HTTLPR.

the long allele when maternal prenatal anxiety was low. At the same time, though, the simple slopes in Figure 1 do suggest a slight trend for a cross-over interaction consistent with differential susceptibility. Follow up analyses revealed that infants with short alleles had lower scores in negative emotionality (M = -.31, SD = 2.05) when mothers did not report anxiety during pregnancy compared to infants homozygous for the long alleles (M = -.21, SD = 2.28), but that this difference was not significant ($t_{(843)}$ = 1.29, p = .26).

Discussion

As hypothesized, 5-HTTLPR moderated the effects of maternal anxiety during pregnancy on infant negative emotionality at six-months postpartum. The hypothesized association between higher levels of maternal anxiety during pregnancy and higher levels of infant negative emotionality proved strongest for individuals with two short alleles, weakest for individuals with two long alleles and intermediate for heterozygotes. In order to investigate whether the significant GXE interaction was more consistent with a diathesis-stress or differential-susceptibility framework, we applied the criteria for the testing of differential susceptibility stipulated by Belsky et al. (17). The criteria that the susceptibility factor (i.e., 5-HTTLPR) be unrelated to predictor and outcome variables was met in that there were no significant associations between, respectively, 5-HTTLPR and maternal anxiety or between 5-HTTLPR and infant negative emotional-

ity. At the same time, however, the graphical display of simple slopes linking prenatal anxiety and infant negative emotionality failed to document a clear cross-over pattern, a further criterion for differential susceptibility; in other words, the interaction under consideration chronicled more a diathesis-stress than differential-susceptibility process of environmental action. This could be the result of reliance on a contextual predictor, prenatal maternal anxiety, whose positive pole merely reflected the absence of an adverse condition, not, say, the presence of a development facilitating positive one. A contextual variable capturing the range of prenatal environmental influences from negative to positive and not just the presence and absence of adversity as in the current analysis might have revealed a cross-over interaction of the kind anticipated by the differential susceptibility framework.

According to the fetal programming hypothesis, the fetus adapts its phenotype to the anticipated postnatal environment based on maternal cues regarding the quality of the outside world (1, 2, 36) in order to function optimally in that specific environment. Gluckman and Hanson (1) make reference to "predictive adaptive responses" which, if the actual environment ends up being different from the one anticipated, generates a mismatch between the programmed phenotype and environment and, consequently, proves dysfunctional rather than adaptive (36). This raises the question whether prenatal-stress effects on sequelae like infant negative emotionality should be regarded as adaptive or maladaptive. Here we entertain the former possibility.

Infant difficult temperament is generally considered a risk factor for and precursor of a range of problematic outcomes (e.g., 37, 38). However, Belsky's (15, 16) reconceptualization of difficult temperament as a marker for developmental plasticity stipulates that highly negatively emotional infants and children have an especially sensitive nervous system and thus are not simply more vulnerable to adversity but also more likely to benefit from enriching and supportive environmental influences. Bradley and Corwyn (39) and Pluess and Belsky (40, 41) provide evidence to this effect with respect to the quality of both parenting and child care. Recently, numerous related findings have been reviewed by Belsky and Pluess (13).

Therefore, prenatal programming of negative emotionality as chronicled in the current study, especially in the case of infants carrying at least one short allele of the 5-HTTLPR, may itself represent an adaptive response: (a) Stressful environmental experiences during pregnancy contribute to (b) the fetal programming of developmental plasticity––demarcated by negative emotionality/difficult temperament––as a means of (c) enhancing the organism's adaptation to the postnatal environment. For example, negative emotionality may sensitize children to carefully observe a potentially threatening postnatal environment and/or help them to regain attention from caregivers that may be distracted by other concerns. It is thus proposed that the association between prenatal maternal anxiety and infant temperament reflects adaptive prenatal program-

ming of postnatal plasticity (42). The current study provides new evidence for such fetal programming of infant temperament but further suggests that such fetal programming effects differ as a function of genotype with the effect of prenatal anxiety on negative emotionality––and therefore hypothetically developmental plasticity––being strongest in individuals carrying the 5-HTTLPR short allele. However, whether negative emotionality moderates postnatal environmental influences in the current sample as the differential susceptibility hypothesis would predict remains to be tested.

This research is not without limits. Consider first that the interaction effect detected was statistically significant but very small, which was due mostly to the fact that maternal prenatal anxiety exerted no apparent effect on negative emotionality for infants homozygous for the long allele. In contrast, for infants carrying two copies of the short allele, maternal prenatal anxiety explained up to three percent of the variance in infant negative emotionality, thereby, implying clinical significance. The second limitation of this inquiry is that infant negative emotionality was based exclusively on maternal report; additionally, ethnicity of the sample was restricted to those of Dutch ancestry. Whether the discerned association between maternal anxiety during pregnancy and infant negative emotionality, especially as moderated by 5-HTTPLR, can be replicated using behavioral measures of negativity and/or in samples comprised of other ethnic groups remains to be determined. Attention needs also to be drawn to the fact that mothers with psychiatric disorders were not identified or excluded from the study, a limitation that would seem to be mitigated by the statistical controls instituted for postnatal depression and anxiety; Finally, it must be appreciated that the study design was correlational, thereby limiting the confidence that can be placed in any causal inferences drawn. Conceivably, for example, the association between maternal anxiety and infant temperament could be an artifact of shared genes and thus heritability, with mothers more easily distressed during pregnancy bearing children who inherit the same propensity to experience stress more readily than others (43, 44).

In conclusion, the work presented herein provides first empirical evidence for the hypothesis that effects of fetal programming are moderated by the 5-HTTLPR. The association between maternal anxiety during pregnancy and negative emotionality in early infancy was significant in infants carrying one or more copies of the short allele, but not in those homozygous for the long allele. Consequently, the 5-HTTLPR short allele may increase vulnerability to adverse environmental influences as early as the fetal period.

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Chapter 5.2

The Generation R Study; a study of the 5-HTTLPR gene by environment interaction from fetal life onwards.

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Adapted from The Generation R Study; a review of design, findings to date and a study of the 5-HTTLPR gene by environment interaction from fetal life onwards.

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ABSTRACT

Objective To examine within Generation R whether the functional polymorphism (5-HTTLPR) in the promoter of the serotonin transporter gene interacts with prenatal maternal chronic difficulties, prenatal maternal anxiety or postnatal maternal anxiety to influence child emotional development. **Method:** 2136 Northern European children were genotyped for 5-HTTLPR and rs25531. Mothers reported chronic difficulties and anxiety symptoms at 20 weeks pregnancy and when the child was 3 years old. Child emotion recognition was observed at 3 years, and child emotional problems were assessed with the CBCL/1½-5 at 5 years. **Results:** There were consistent main effects of maternal difficulties and anxiety on child emotional problems, but no main effect of 5-HTTLPR. Moreover, children with the s allele were at increased risk for emotional problems if their mothers reported prenatal anxiety symptoms (beta 2.02, $p < 0.001$) or postnatal anxiety symptoms (beta 1.64, $p < 0.001$). Also, in children of mothers with prenatal anxiety symptoms, the s allele was associated with less accurate emotion-matching (beta -0.11, $p = 0.004$). **Conclusions:** This population-based study shows that vulnerability due to 5-HTTLPR is not specific for certain adverse exposures or severe events, but suggests that the small effects of gene-environment interaction on emotional development become manifest early in life.

INTRODUCTION

A decade ago, Caspi and colleagues reported that carriers of the short (s) allele of the common functional polymorphism (5-HTTLPR) in the promoter region of the serotonin transporter gene (*5-HTT, SLC6A4*) are at higher risk to develop depression following stressful life-events (SLE) or childhood maltreatment than adults homozygous for the long (l) allele.[1] This finding represented important epidemiologic support for gene-environment interaction (GxE), and was followed by numerous replication attempts. The first two meta-analyses [2-3] (k=5 and k=14) did not show evidence for a significant interaction effect of 5-HTTLPR and SLE on depression. Karg and colleagues used a different statistical approach for their meta-analysis[4] (k=54) and allowed for chronic diseases as indicators of stress. Unlike the former, this latter meta-analysis found evidence for moderation by 5-HTTLPR on the relation between stress and later development of depression.

This inconsistency fuelled a debate about the validity of GxEs.[5] Variation in inclusion criteria, different statistical methods, additional allelic variation (rs25531), heterogeneity of subjects and choice of phenotype may explain divergent results.[6-7] It was suggested that the moderation by 5-HTTLPR was specific to certain stressors, such as childhood maltreatment or severe illness, and to a lesser extent to compilations of stressful life-events.[8] It was also posited that the interaction effect between 5-HTTLPR and childhood maltreatment would be specific to persistent major depression.[9] Furthermore, it was reported that studies using observational data to assess the environment more often replicated the GxE than studies using self-report measures.[8] The current study further explores early moderation of environmental effects by 5-HTTLPR on child development within the context of this debate.

The 5-HTTLPR polymorphism plays an important role in stress-sensitivity across species. The s allele has been associated with lower transcriptional efficiency of the promoter and consequently reduced serotonin transporter availability.[10] Neuroimaging studies showed that the s allele is associated with increased amygdala reactivity and decreased amygdala-prefrontal functional coupling when viewing threatening stimuli.[8, 11] The findings by Lau and colleagues suggest that the effect of 5-HTTLPR on amygdala reactivity may differ in healthy individuals compared to patients. Healthy s-allele carriers showed increased amygdala reactivity in response to fearful faces, whereas in patient the l-allele was associated with increased amygdala response to fearful and happy faces . [12]

Serotonin is one of the earliest neurotransmitters, appearing at approximately 5 weeks of gestation.[13] Consequently, 5-HTTLPR may influence early neurodevelopment and brain function. [8, 14] It has been shown that stress during pregnancy affects the development of the brain areas involved in emotional development; i.e. the hippocampus, the frontal lobe and the amygdale.[15] These prenatal programming effects may lead

to changes in amygdala volume and could modify the trajectories of connections. Possibly, these effects are further moderated by 5-HTTLPR. To better understand early moderation by 5-HTTLPR and its consequences, it is essential to study GxE during the fetal period and early childhood.[8] We previously showed that 5-HTTLPR interacts with prenatal maternal anxiety to affect child negative emotionality in infants.[16] Here, we further explore the effect of 5-HTTLPR during early development in a broader context and performed additional analyses to test the validity of the results. First, we explored whether 5-HTTLPR moderates the effects of prenatal maternal chronic difficulties or anxiety symptoms on child emotional problems. We contrasted the interaction effects observed for maternal and paternal prenatal anxiety to examine direct intrauterine effects. Second, we studied whether 5-HTTLPR interacts with postnatal maternal anxiety symptoms on the risk of child emotional problems. Third, we used an observational measure of emotion-matching accuracy to explore the interaction of 5-HTTLPR and maternal anxiety symptoms on children's ability to recognize emotional faces. Philips et al. postulate that emotion perception involves three interrelated processes, the recognition of emotionally salient cues, emotional behavioral responses and the regulation of affect.[17] We studied the first process by utilizing facial expressions as emotionally salient cues. This follows a common approach in emotion processing research of typically developing children. Moreover, deficits in these abilities have repeatedly been linked to psychiatric problems at different ages. [18] Genetic factors contribute to influence facial expression recognition [19-20], possibly through the alteration of key neurotransmitter systems such as the serotonin system. [21] Finally, we tested whether any observed GxEs in Northern European children were consistent across ethnicities in subgroups of children of Turkish, Moroccan and Surinamese descent.

METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from fetal life onwards in Rotterdam, the Netherlands. The Generation R Study has previously been described in detail.[22-23] All children were born between April 2002 and January 2006. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from parents of the participating children.

Population of analysis

In genetic analyses, population stratification can increase the rate of false positive findings in heterogeneous samples like the Generation R Cohort. The GWAS data in our multi

ethnic cohort made it possible to compare and complement the self reported ethnicity of participants with genetic information about descent. We firstly selected children of Northern European descent, which was determined by principle component analyses of genome wide association data, as described previously.[22] Principle component analyses yield factors that can be interpreted as the direction which maximizes the variance of the sample while being uncorrelated to previous components. From the group of 3410 children with a self-reported North-European ethnicity, 2841 (83%) children of Northern European descent were identified. Within these children, information about the 5-HTTLPR was available in 2589 children.

In 2136 (82.5%) of these 2589 eligible children, information was available on maternal chronic difficulties, maternal anxiety and child emotional problems. To test for generalizability, we included 581 Turkish, Moroccan and Surinamese children. Additional analyses were performed in a smaller sample of children participating in the Generation R Focus Study,[23] in which observational data are available. Information on 5-HTTLPR allele status and prenatal maternal anxiety symptoms was available for 617 children. At age 3 years, 570 of these children also had data on emotion-matching accuracy.

The study population thus comprised 2136 children of Northern European descent, of whom 570 children also participated in the Focus Study. The generalizability of our findings was tested in 228 Turkish, 146 Moroccan and 207 Surinamese children.

Genotyping

DNA was derived from cord blood samples at birth. The 43-base pair insertion/deletion in 5-HTTLPR and rs25531 were genotyped using Taqman allelic discrimination. The forward primer was 5'-GGCGTTGCCGCTCTGAATGC-3', and the reverse primer was 5'-GAGGGACTGAGCTGGACAACCAC-3'.[24] Upstream of the *5*-HTTLPR, prs25531 results in two functional variants of the l-allele; l_A and l_G. [25] The l_G variant may be associated with lower *5-HTT* expression.[24] Reactions were performed in a 384-wells format in a total volume of 5 ul containing 2 ng DNA, 120 nM FAM-probe, 80 nM VIC-probe, PCR primers (100 nM each), dimethyl sulfoxide (DMSO) (4% by volume), and 1 x genotyping master mix (Applied Biosystems Inc.). PCR cycling consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 96° C and annealing and extension for 90 seconds at 62.5° C. Signals were read with the Taqman 7900HT (Applied Biosystems Inc.) and analyzed using the sequence detection system 2.3 software. To check for potential contamination with maternal blood, gender was determined in male participants. Contamination occurred in < 1% of cases. To evaluate genotyping accuracy, 225 random samples were genotyped twice. No discrepancies were found. Genotyping information about DRD4 and MAO-A are presented in the supplementary material S6 (see S6).

Maternal Chronic Difficulties and Anxiety Symptoms

Prenatal chronic difficulties of the mother were assessed at 20 weeks pregnancy with a validated self-report questionnaire.[26] In this 13-item questionnaire, long-lasting situational and relational difficulties that occurred in the preceding year were measured on a five-point scale, e.g. "Have you had any financial problems in the past year?"

Prenatal and postnatal parental symptoms of anxiety were assessed at 20 weeks pregnancy and 3 years after birth using the Brief Symptom Inventory (BSI), a validated self-report measure of general psychopathology with 53 items to be answered on a five-point scale, ranging from "0=not at all" to "4=extremely".[27-28] The current study focused on the anxiety scale, which consists of 6 items, e.g. "nervousness or shaking inside". The Cronbach's alpha was 0.59 for the six anxiety items, and 0.92 for all items. Postnatally, the alpha was 0.65 for the anxiety symptoms and 0.84 for all items of the BSI.

Child Emotional Problems and Emotion Processing

First, we studied the effect of gene-environment interaction on child emotional problems measured with The Child Behavior Checklist/1½-5 (CBCL/1½-5) [29]. The 99 items are scored on a three-point scale, ranging from "0 = not true" to"2 = very true". We used the Internalizing scale to define child emotional problems at the age of 5 years. The Internalizing scale (36 items) covers the Emotionally Reactive, Anxious/Depressed, Somatic Complaints, and Withdrawn syndrome scales.

Second, we studied the effect of gene-environment interaction on child emotion recognition. Child emotion recognition was assessed using a computerized *emotion-matching task*.[30] Color images depicting four basic emotions (happiness, sadness, anger, and fear) were presented on a screen and children were required to match the emotion of a target face presented at the top of the computer screen with one of two faces presented below. In total, there were 16 trials with two female and two male identity pairs and four basic emotions. To control for the effects of basic matching ability a *shape-matching task* was included prior the emotion-matching task. Similar paradigms have been used previously.[31] Accuracy on both tasks was calculated as the ratio of correct responses to the number of trials attempted.

Covariates

Gestational age was established by fetal ultrasound examinations. Gestational age, Apgar score, birth weight, and gender were obtained from midwife and hospital registries at birth; information about maternal age , parity, educational level and child age at the time of the assessment of behavior was obtained by questionnaire.

Statistical analysis

We examined selective non-response by comparing characteristics between mothers and children included and those excluded from this study using chi-square statistics,

independent t-tests, and Mann Whitney U-tests. Using the same tests, we also compared characteristics of mothers and children in this study by 5-HTTLPR genotype. The correlation between the predictors was calculated with the Spearman's correlation coefficient for non-parametric variables (two-tailed). Power calculations were performed using Quanto. [32] These calculations were based on continuous measurements in 2136 independent individuals, assuming an additive genetic model.

Gene-environment interaction

Before computing product terms for the interactions the predictors were centered. We studied main effects of 5-HTTLPR, prenatal maternal chronic difficulties, prenatal and postnatal maternal anxiety symptoms on child emotional problems. To test our hypothesis, we first tested for the interaction of 5-HTTLPR with prenatal maternal chronic difficulties, and the interaction of 5-HTTLPR with prenatal maternal anxiety symptoms on child emotional problems. In this analyses we covaried for the interaction of postnatal symptoms with 5-HTTLPR to examine specificity of any intrauterine exposure effect. To evaluate possible confounding, we also tested for interaction between 5-HTTLPR and prenatal *paternal* anxiety symptoms on child emotional problems.

Second, we tested for interaction between 5-HTTLPR and postnatal maternal anxiety symptoms on child emotional problems. Again, to test specificity, we covaried for prenatal exposure.

Third, we explored the specificity of this GxE to 5-HTTLPR by examining moderation of the dopamine D4 receptor gene repeat (DRD4 48bp VNTR) and the polymorphism in the promoter of the monoamine oxidase A gene (MAO-A) on the relation between maternal anxiety symptoms as well as chronic difficulties and child emotional problems. Based on a review of previous studies, we expected to find that carriers of the DRD4 7 repeat (DRD4 7R) with mother who had anxiety symptoms were more likely to have emotional problems compared to non-carriers. [33] Although these studies, however, focused typically on externalizing problems. We expected to find a decreased risk of emotional problems in carriers of the high activity MAO-A who had mothers with anxiety symptoms compared to carriers of the low activity MAO-A. [34]

Fourth, we examined the effect of interaction between 5-HTTLPR and prenatal maternal anxiety symptoms on child emotion-matching accuracy. Linear regression analyses were run under the assumption of an additive genetic model. Logistic regression analyses were run to present results for a three-categorical genetic model with the l/l genotype as reference category. We repeated the analyses including rs25531, for which we assigned individuals to three groups based on functional similarity of the s-allele and the l_g allele; high expression (l_a/l_a), intermediate expression (l_a/l_g or s/l_a) and low expression (l_g/s or s/s).

Fifth, we tested generalizability by performing a meta-analysis with Northern European and non-European children, using the inverse Z score method assuming random effects (R 2.14.0).

Missing data

All models were adjusted for maternal age, educational level, smoking during pregnancy, parity, gestational age, gender, Apgar score, and child age. To test for independent effects, the analyses were also adjusted for the interaction term of other predictors. Missing data occurred in this longitudinal project due to attrition and failure to complete all assessments as follows: maternal education (0.02%), smoking during pregnancy (0.004%), Apgar score (0.1%), parity (0.002%), prenatal maternal anxiety (11%) and postnatal maternal anxiety (19%), prenatal paternal anxiety (17%), and postnatal paternal anxiety (26%), and were imputed using multiple imputation. Results were averaged across five imputed data sets. The level of significance for all analyses was set at $\alpha = .05$.[35]

Non-response analysis

Children (n=453) excluded from our study more likely had younger (29.9 vs. 31.9 years, $t = 8.37$, $p < 0.001$) and less highly educated mothers (28.7 vs. 39.8%, $\chi 2 = 18.7$(1df), $p < 0.001$), that more often smoked during pregnancy (33.9 vs. 20.0%, $\chi 2 = 40.4$(1df), $p < 0.001$) than mothers of children included in our study (n=2136). The distribution of gender and parity did not differ between the two groups.

RESULTS

Of the 2136 children of Northern European descent, 719 (33.7%) were homozygous for the l-allele, 1029 (48.2%) were heterozygous, and 388 (18.2%) were homozygous for the s-allele. Genotypic distribution conformed to Hardy-Weinberg equilibrium (HWE), $\chi^2 (2) = 0.55$, $p = 0.76$.

Mother and child characteristics are presented in Tables S1 and S5. The distribution of the characteristics did not differ according to 5-HTTLPR status which suggests that the s-allele is not significantly correlated to these characteristics. The correlation between the environmental risk factors ranged from 0.18 (prenatal maternal chronic difficulties and postnatal anxiety symptoms) to 0.48 (prenatal maternal chronic difficulties and prenatal maternal anxiety symptoms). 5-HTTLPR was not significantly correlated with prenatal chronic difficulties, prenatal anxiety or postnatal anxiety of the mother. Child emotional problems scores ranged from 0 to 49 (mean 5.2, standard deviation 5.3). The study of emotional problems had 0.89 power to detect gene-environment interaction with an explained variance of 1%.[36] The power was 0.86 to detect a GxE effect explaining 0.4% of the variance. The high power is likely the result of our sample size and the high frequency of the s-allele. [37] Due to its smaller sample size, the study on child emotional regulation was not sufficiently powered (0.70) to find a significant GxE with an explained variance of 0.01.

We first tested for interaction between 5-HTTLPR and prenatal maternal stressors on child emotional problems (Table 1). Regression analyses indicated no genetic main effect of 5-HTTLPR on child emotional problems. Prenatal maternal chronic difficulties and anxiety symptoms predicted child emotional problems. 5-HTTLPR moderated the association between maternal chronic difficulties and child emotional problems, but this effect was not independent of prenatal maternal anxiety symptoms (beta 0.07, se 0.09, $p = 0.44$). Also, 5-HTTLPR moderated the effect of prenatal maternal anxiety symptoms on emotional problems (beta 2.02, se 0.55, $p = 0.001$). Compared to l/l carriers, l/s car-

Table 1 Results of the interaction of *5-HTTLPR* status with prenatal chronic difficulties, prenatal anxiety symptoms and postnatal anxiety symptoms of mothers on the risk for emotional problems in preschool children.

		Child Emotional Problems at 5 years (n=2136)[a]					
Prenatal maternal chronic difficulties		beta	se	*p*	R^2	ΔR^2	ΔF
step 1	prenatal chronic difficulties	0.19	0.06	0.003	0.099	-	-
step 2	*5-HTTLPR*	0.05	0.16	0.730	0.099	0.000	0.69
step 3	*5-HTTLPR* x chronic difficulties	0.06	0.08	0.434	0.099	0.000	0.369
per genotype							
	l/l	ref					
	l/s	0.10	0.12	0.407			
	s/s	0.20	0.16	0.207			
Prenatal maternal anxiety symptoms							
step 1	prenatal maternal anxiety (pre anx)	3.11	0.38	<0.001	0.098	-	-
step 2	*5-HTTLPR*	0.11	0.16	0.494	0.099	0.000	0.42
step 3	*5-HTTLPR* x pre anx	2.02	0.55	0.001	0.109	0.010	24.71
per genotype							
	l/l	ref					
	l/s	2.06	0.87	0.018			
	s/s	3.32	1.11	0.003			
Postnatal maternal anxiety symptoms							
step 1	postnatal maternal anxiety (post anx)	2.21	0.60	0.001	0.107	-	-
step 2	*5-HTTLPR*	0.07	0.16	0.656	0.107	0.000	0.23
step 3	*5-HTTLPR* x post anx	1.64	0.71	0.023	0.109	0.002	5.15
per genotype							
	l/l	ref					
	l/s	3.47	1.15	0.004			
	s/s	3.12	1.50	0.040			

[a]Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, age child at behavioral assessment, 5-HTTLPRxprenatal maternal anxiety symptoms or 5-HTTLPRx postnatal maternal symptoms of anxiety.

riers (beta 2.06, se 0.87, $p = 0.018$) and s/s carriers (beta 3.32, se 1.11, $p = 0.003$) were at an increased risk for child emotional problems. To further evaluate the possibility of an intrauterine effect and the role of confounding, we also examined the effect of interaction between 5-HTTLPR and prenatal *paternal* anxiety symptoms on child emotional problems. We found no significant interaction between 5-HTTLPR and prenatal anxiety symptoms of the father (beta 0.54, se 0.82, $p = 0.51$) (see Table S2).

Next, we tested for interaction between 5-HTTLPR and postnatal maternal anxiety symptoms on child emotional problems. 5-HTTLPR moderated the association between postnatal anxiety symptoms emotional problems (beta 1.64, se 0.71, $p= 0.023$). Compared to l/l carriers, l/s carriers (beta 3.47, se 1.15, $p = 0.004$) and s/s carriers (beta 3.12, se 1.50, $p = 0.04$) were at an increased risk for child emotional problems. The effect was independent of the interaction between 5-HTTLPR and prenatal maternal anxiety symptoms. Importantly, the reverse was also true; the interaction of prenatal maternal anxiety symptoms and 5-HTTLPR was independent of the interaction between 5-HTTLPR and postnatal anxiety symptoms. The regression slopes are plotted in Figure 1.

To further study the specificity of this interaction to 5-HTTLPR, we also ran the analyses studying genetic variants the DRD4 gene and the MAO-A gene. MAO-A interacted with prenatal maternal anxiety symptoms resulting in a decreased risk of child emotional problems (beta -2.98, se 0.95, $p = 0.002$) (see Table S3). The significant interaction between the DRD4 7R and high levels of prenatal maternal anxiety symptoms and the lower risk of emotional problems was not in the hypothesized direction (beta -1.44, se 0.67, $p = 0.03$). Neither of these two genetic variations modified the effect of maternal chronic difficulties or postnatal anxiety on child emotional problems.

Then, we studied the interaction effect of 5-HTTLPR with prenatal maternal anxiety symptoms on children's emotion-matching accuracy. There were no main effects of 5-HTTLPR (beta -0.01, se 0.01, $p = 0.36$) or prenatal maternal anxiety symptoms (beta -0.02, se 0.04, $p = 0.66$) on child emotion-matching accuracy. 5-HTTLPR significantly interacted with prenatal maternal anxiety symptoms to influence child emotion-matching accuracy (beta -0.12, se 0.05, $p = 0.011$). Compared to l/l carriers, s/s carriers matched emotions less accurately if exposed to higher levels of maternal anxiety before birth. This effect was independent of the interaction effect between 5-HTTLPR and postnatal maternal anxiety symptoms. In Figure 2, regression slopes are plotted. Allelic variance at rs25331 did not change the results nor improved the model fit (see Table S4).

We then examined the effect of these interactions in the three largest ethnic minorities in the Generation R cohort. Genotype distributions in Turkish and Moroccan children conformed to HWE (Turkish children $\chi^2_{(1)} = 0.45$, $p > 0.05$, Moroccan children $\chi^2_{(1)} = 0.01$, $p > 0.05$), but deviated in Surinamese children ($\chi^2_{(1)} = 5.33$, $p < 0.05$). This deviation is likely due to large heterogeneity in this population. Hence, we limited the analyses to Turkish (n=228) and Moroccan children (n=146). In Turkish and Moroccan children, the interaction effects were not-significant. The interaction between 5-HTTLPR and postna-

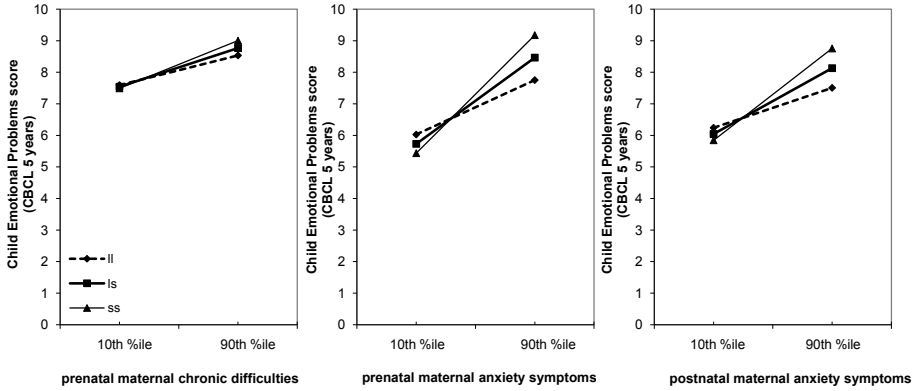

Figure 1. The relation between prenatal maternal chronic difficulties, prenatal maternal anxiety symptoms, and postnatal maternal anxiety symptoms and child emotional problems according to 5-HTTLPR genotype. The 5-HTTLPR does not moderate the relation between prenatal maternal chronic difficulties (beta 0.06, se 0.08 , $p = 0.434$) and child emotional problems. The relation between prenatal maternal anxiety symptoms (beta 2.02, se 0.55, $p = 0.001$), and postnatal maternal anxiety symptoms (beta 1.64, se 0.71, $p = 0.023$) and child emotional problems depends on 5-HTTLPR status. Coefficients come from a multiple linear regression model adjusted for children's age, sex, gestational age at birth, maternal age, education, parity, smoking during pregnancy, 5-HTTLPR*prenatal maternal anxiety symptoms, and 5-HTTLPR*postnatal maternal anxiety symptoms. The values at the x-axis represent the 10th and 90th percentile score on prenatal maternal anxiety symptoms (centered). CBCL; child behavior checklist.

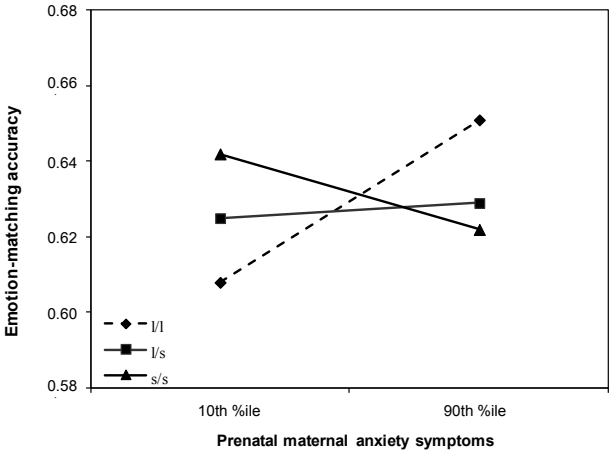

Figure 2. The relation between prenatal maternal anxiety symptoms and emotion-matching accuracy according to 5-HTTLPR genotype. The relation between prenatal maternal anxiety symptoms and emotion-matching accuracy depends on 5-HTTLPR status (beta -0.12, se 0.05 , $p = 0.011$). Coefficients come from a multiple linear regression model adjusted for children's age, sex, gestational age at birth, shape-matching accuracy, maternal age, education, parity, smoking during pregnancy, 5-HTTLPR*postnatal maternal anxiety symptoms. The values at the x-axis represent the 10th and 90th percentile score on prenatal maternal anxiety symptoms (centered)

Table 2 Results of the interaction of *5-HTTLPR* status with prenatal chronic difficulties, prenatal anxiety symptoms and postnatal anxiety symptoms of mothers on the risk for child emotional problems in Turkish, Moroccan and Northern European children.

		Child Emotional Problems at 5 years								
		Turkish children[a] n=228			Moroccan children[a] n=146			Meta-analysis Northern European, Turkish and Moroccan children[a] n=2510		
		beta	se	*p*	beta	se	*p*	beta	se	*p*
Prenatal maternal chronic difficulties										
step 1	prenatal chronic difficulties	-0.06	0.72	0.78	0.01	0.17	0.96			
step 2	*5-HTTLPR*	-0.11	0.72	0.88	-2.09	0.80	0.01			
step 3	*5-HTTLPR* x chronic difficulties	0.4	0.27	0.62	0.06	0.23	0.79	0.07	0.07	0.37
Prenatal maternal anxiety symptoms										
step 1	prenatal maternal anxiety (pre anx)	1.19	0.96	0.21	1.55	1.00	0.12			
step 2	*5-HTTLPR*	-0.09	0.71	0.90	-1.88	0.81	0.02			
step 3	*5-HTTLPR* x pre anx	1.73	1.46	0.24	-2.82	1.74	0.12	0.67	0.07	0.36
Postnatal maternal anxiety symptoms										
step 1	postnatal maternal anxiety (post anx)	2.36	1.67	0.16	0.93	1.82	0.61			
step 2	*5-HTTLPR*	-0.11	0.71	0.88	-2.01	0.79	0.01			
step 3	*5-HTTLPR* x post anx	2.23	2.84	0.44	1.90	3.30	0.57	1.68	0.67	0.01

[a]Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, age child at behavioral assessment, 5-HTTLPRxprenatal maternal symptoms of anxiety or 5-HTTLPRxpostnatal maternal symptoms of anxiety.

tal maternal anxiety symptoms in Turkish children went in the same direction and within the confidence intervals of effects in the Northern European children (Table 2).

DISCUSSION

We studied whether children with the s allele of 5-HTTLPR were more vulnerable to prenatal maternal chronic difficulties, prenatal maternal anxiety symptoms or postnatal maternal anxiety symptoms than children with the l allele. Our results suggest that during fetal life and early childhood the effect of maternal anxiety is moderated by child 5-HTTLPR, which places children at increased risk to develop emotional problems and affects the basic ability to process emotions.

Because of the considerable controversy surrounding how best to understand GxE studies of 5-HTTLPR it is important to place our findings in the context of the debate

around the importance of 'main effects'. Others have posited that the study of gene-environment interaction should be conditional on the presence of a genetic main effect on the phenotype.[3] We do not agree. In our study, like others, we did not find a main effect of 5-HTTLPR on child emotional problems or emotion-processing accuracy. If the genetic effect is only apparent in the high range of the environmental stressor and not in the low range, gene-environment interaction may be present without a genetic main effect.[6] An indication for this effect can be seen in the figures illustrating the results of our study, which shows a cross-point in the range of variance in the environmental predictor. There are a variety of potential biological mechanisms to explain both our findings and rational. In the future, we intend to testthat 5-HTT is liable to methylation under environmental stress, which also influences the efficiency of the gene.[38] For this reason we argue that the lack of main effect is not an obstacle to understanding GxE relations in our study.

This study examined moderation of exposure to maternal anxiety by 5-HTTLPR during pregnancy and early childhood. In line with our hypothesis, we found that 5-HTTLPR interacts with prenatal maternal anxiety symptoms to influence the risk for child emotional problems. The interaction effect of 5-HTTLPR and chronic difficulties was accounted for by that of 5-HTTLPR and prenatal anxiety symptoms. These two measures are correlated, and it may be that maternal anxiety impacts to a greater degree on maternal physiology and the fetal environment than chronic difficulties. Further support for a direct intrauterine interaction effect of 5-HTTLPR and maternal anxiety symptoms during pregnancy comes from the absence of any interaction effect of 5-HTTLPR by fathers' prenatal anxiety symptoms.

5-HTTLPR also moderated the relation between postnatal maternal anxiety symptoms and child emotional problems. Plausibly, different mechanisms account for independent prenatal and postnatal interaction effects. In rats, it has been shown that *5-HTT* interacts with prenatal maternal stress to specifically affect offspring's glucocorticoid receptor gene mRNA and corticosterone response, whereas the interaction between *5-HTT* and postnatal stress specifically influenced behavioral responses.[39] Also in humans, it has been recognized that prenatal and postnatal adversity exert different effects on the developing brain.[15] During pregnancy, maternal stress hormone levels may affect the development of the amygdala, the hippocampus and the prefrontal cortex, which are involved in regulating the HPA-axis. We found an interaction effect between 5-HTTLPR and prenatal maternal anxiety on children's ability to recognize emotional expressions. This finding provides indirect evidence for the potential influence of 5-HTTLPR on the development of specific brain networks involving emotion recognition.

Despite the regular contact most fathers have with their children during the preschool years, 5-HTTLPR did not significantly moderate the relation between fathers' anxiety symptoms during the early preschool years and child emotional problems. During early childhood, fathers scored lower on anxiety symptoms than mothers, which may explain

the absence of an interaction effect in fathers. It might also be that fathers spend less time with their children, and thus may have less impact on their child's development.

The DRD4 7R and high activity MAO-A gene variant also moderated the effect of prenatal maternal anxiety symptoms on child emotional problems. However, the results for DRD4 were not in line with our hypothesis. The results for MAO-A were less consistent than our findings with 5-HTTLPR, which also interacted with postnatal maternal symptoms on the risk of emotional problems as hypothesized.

To limit the possible bias due to population heterogeneity, we only included children of Northern European descent based on a principle component analysis of GWAS data. The analyses across Turkish and Moroccan children suggests that the observed moderation of postnatal anxiety effects by 5-HTTLPR can –to some extent– be generalized to other ethnicities.

Last, we did not find evidence to suggest that variance at rs25531 had a large impact on our results. Clearly, this impact of rs25531 also depends on the frequency of the G allele in a specific sample or population. Furthermore, there is no consensus about the genetic model underlying the effect of 5-HTTLPR. Overall, our findings indicate an additive effect of the s allele suggesting that heterozygosity is also associated with an increased risk for child emotional problems.

Although our study has notable strengths, including the large sample size and prospective data assessments, there are some limitations that need to be considered. First, we relied on self report of mothers and fathers on anxiety symptoms. These symptom scores yield a relatively crude measure that cannot be translated into a clinical diagnosis of anxiety disorder. However, our results show that even these subclinical levels of anxiety influence child emotional development. Second, we did not explore the effect of the interaction between maternal 5-HTTLPR status and the environment on child emotional development. Kistner-Griffin and colleagues found that the maternal 5-HTTLPR l-allele was associated with autism spectrum disorder in the offspring. [40] Possibly, maternal 5-HTT influences fetal brain development through metabolic pathways in the placenta. [41] Hence, to gain further insight in the underlying mechanisms it would be of interest to further explore moderation by maternal 5-HTTLPR during pregnancy on child emotional development.

Brown and Harris recently hypothesized that the interaction between 5-HTTLPR and early childhood adversity may affect neurodevelopment and brain function, which could result in a life-time increased risk to develop psychopathology. Moreover, the authors suggested that these early effects on neurodevelopment may even account for the risk of depression associated with the interaction between 5-HTTLPR and SLE during adulthood.[14] This suggests that gene-environment interaction during adulthood is observed particularly in those s-allele carriers that were exposed to environmental adversity during childhood. Our findings support the first part of this hypothesis by

reporting interaction effects of 5-HTTLPR with the prenatal environment and early childhood on child emotional development.

In complex traits, such as emotional problems, large effects of one polymorphism in interaction with the environment are rare. Moreover, the small effects of 5-HTTLPR may be plausible from an evolutionary perspective. In rhesus monkeys, s allele carriers showed delayed early neurobiological development, impaired serotonergic functioning, HPA-axis reactivity and aggression, but only if they were peer reared. If they were reared by their mother, the s allele carriers showed better behavioral outcomes than l allele carriers.[42] Next to an increased sensitivity to stress, the s allele has been associated with improved cognitive function, such as decision making and social cognition.[21, 43] Hence, s allele carriers seem very attentive to the environment, capable to process information under stress, which likely contributed to adaptive success and survival of the species.[42-43] It has been suggested that 5-HTTLPR acts as a plasticity gene, influencing the individual´s susceptibility to the environment. [44-45]We explored moderation of 5-HTTLPR within the diathesis-stress framework. Future research should further explore possible differential effects of 5-HTTLPR according to the quality of the environment. This would include the assessment of a positive aspect of the environment other than just the absence of anxiety symptoms in our study.

In sum, our findings suggest that *5-HTTLPR* moderates the impact of the environment on the fetus and the child, influencing emotion processing and the risk for emotional problems later in life. The impact of moderation of *5-HTTLPR* does not lie in the magnitude of the effect, but lies in the non-specificity and its generalizability across ethnicities. Future studies are needed to show if these small effects in large studies are replicable or further converge to the null.[46]

SUPPLEMENTARY MATERIAL

SM Table 1 Sample characteristics by *5HTTLPR* genotype (n=2136)

	Child *5HTTLPR*				
	l/l (n=719)	l/s (n=1029)	s/s (n=388)	Test statistic [a, c]	*p* value
Mother					
Age at intake (years)	32.2(3.9)	31.8(4.0)	31.8(4.0)	3.01	0.05
Education					
higher education (%)	39.7	41.0	36.7	2.02	0.35
Marital status					
married/living together %	96.4	95.8	96.3	0.56	0.76
Smoking during pregnancy					
never %	79.5	80.0	80.9	0.31	0.81
Child					
Gestational age (weeks) [b]	40.4 (37.4-42.1)	40.4 (37.6-42.1)	40.3 (37.7-42.1)	3.05	0.08
Birth order					
first child (%)	58.1	60.9	61.3	1.75	0.42
Gender					
boys (%)	50.1	52.1	49.2	1.22	0.55

[a]mean (standard deviation) unless otherwise indicated
[b]median (100% range)
[c]with the chi-square statistic for categorical variables, one-way ANOVA (analysis of variance) for normally distributed continuous variables and the Kruskal-Wallis test for non-normally distributed continuous variables.

SM Table 2 Results of the interaction effect of 5HTTLPR with prenatal and postnatal paternal anxiety symptoms on child emotional problems (n=2136).

	Child Emotional problems at 5 years[a]		
	beta	se	*p*
5HTTLPR x prenatal paternal anxiety symptoms[b]			
additive genetic model	0.54	0.82	0.51
per genotype			
l/l	ref		
l/s	0.48	1.14	0.67
s/s	1.26	1.75	0.47
5HTTLPR x postnatal paternal anxiety symptoms			
additive genetic model	-0.53	0.99	0.59
per genotype			
l/l	ref		
l/s	0.08	1.38	0.95
s/s	-1.27	2.07	0.54

[a]Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, age child at behavioral assessment, 5HTTLPRxprenatal paternal symptoms of anxiety or 5-HTTLPRxpostnatal paternal symptoms of anxiety.
[b] centered

SM Table 3 Results of the interaction of MAO-A and DRD4 48bp VNTR status with prenatal chronic difficulties, prenatal anxiety symptoms and postnatal anxiety symptoms of mothers on the risk for emotional problems in preschool children.

	Child Emotional Problems at 5 years		
	beta	se	*p*
MAO-A[b] n=1000			
MAO-A x Prenatal maternal chronic difficulties	-0.24	0.14	0.080
MAO-A x Prenatal maternal anxiety symptoms	-2.98	0.95	0.002
MAO-A x Postnatal maternal anxiety symptoms	1.30	1.41	0.359
DRD4 7R[c] n=2136			
DRD4 x Prenatal maternal chronic difficulties	-0.11	0.09	0.207
DRD4 x Prenatal maternal anxiety symptoms	-1.44	0.67	0.029
DRD4 x Postnatal maternal anxiety symptoms	-0.48	0.90	0.597

[a]Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, age child at behavioral assessment, prenatal maternal anxiety symptoms or postnatal maternal symptoms of anxiety.
[b] analysis were performed in boys only; the beta is the effect estimate for the high MAO-A activity variant (4, n=642) compared to the low activity MAO-A (3, n=358).
[c] assuming an additive genetic model; homozygous without the 7 repeat (n=1394), heterozygous (n=679), and homozygous for the 7 repeat (n=63).

SM Table 4 The moderating effect of variance at rs25531 on the association between prenatal and postnatal maternal risk factors and child emotional problems (n=2033).

	Child emotional problems at 5 years		
	beta	se	*p*
rs25531 x prenatal maternal chronic difficulties			
additive[a]	0.04	0.08	0.642
rs25531 x prenatal maternal anxiety symptoms			
additive[a]	1.90	0.53	0.001
rs25531 x postnatal maternal anxiety symptoms			
additive[a]	2.05	0.76	0.009

Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, age child at behavioral assessment, prenatal maternal symptoms of anxiety/ postnatal maternal symptoms of anxiety.
[a] The distribution of the variation at rs25531 was as follows; s/la 42.6%, s/lg 5.6%, la/la 25.0%, la/lg 8.1%, lg/lg 0.3%, s/s 18.4%. additive genetic model; l_a (n=505) vs. l_a/l_g or s (n=1029) vs. l_g and/or s (n=499), Hardy-Weinberg Equilibrium (HWE), χ^2 (2) = 0.34, p = 0.84

SM Table 5 Subsample characteristics by *5-HTTLPR* genotype (n=570)

	Child *5-HTTLPR*				
	l/l (*n*= 183)	l/s (*n*= 270)	s/s (*n* = 117)	test[a]	*p*
Mother					
Age at intake (years), mean (SD)	32.3 (3.9)	32.12(3.7)	32.3 (3.9)	0.06	0.94
Educational level (% high)	66.9	69.5	68.6	0.37	0.83
Parity (% primiparous)	61.7	58.1	61.5	0.73	0.69
Smoked during pregnancy (% yes)	24.0	19.3	15.4	3.52	0.17
Child					
Age (months), mean (SD)	37.5 (1.3)	37.6 (1.5)	37.6 (1.5)	0.59	0.55
Gender (% boys)	45.9	50.0	47.0	0.80	0.67
Gestational age at birth (weeks), mean (SD)	40.3 (1.3)	40.3 (1.3)	40.2 (1.3)	0.42	0.66
Shape-matching accuracy, mean (SD)	0.91 (0.2)	0.92 (0.2)	0.91 (0.2)	0.12	0.89

[a]with the chi-square statistic for categorical variables, one-way ANOVA (analysis of variance) for normally distributed continuous variables.

SM 6 Genotyping DRD4 48bp VNTR and MAO-A VNTR

Genotyping of the VNTR polymorphism in the DRD4 gene.

Genotyping of the DRD4 48bp VNTR was amplified using primers D4-F-GCGACTACGTGGTCTACTCG and D4-R-AGGACCCTCAGGCCTTG. Reactions were performed in a 384-wells format in a total reaction volume of 10ul containing 10ng DNA, 1 pmol/ul of each primer, 0.4 mM dNTPs, 1 M betaine, 1x GC buffer I (Takara Bio Inc.) and 0.5 U/ul LA Taq (Takara Bio Inc.). PCR cycling consisted of initial denaturation of 1 min at 94 °C, and 34 cycles with denaturation of 30 seconds at 95°C, annealing of 30 seconds at 58°C and extension of 1 minute at 72°C. PCR fragments were size-separated on the Labchip GX (Caliper Life sciences) using a HT DNA 5K chip (Caliper Life sciences). The number of DRD4 repeats was determined using the size of PCR-fragments. To assure genotyping accuracy 225 random samples were genotyped a second time. Three samples (1.3%) gave different genotypes. These discrepancies were specific for the repeats longer than 7. The HT DNA 5k chip was unable to accurately distinguish the 7, 8, 9 and 10 repeat. As the frequency of the 8, 9 and 10 repeat is low; all samples with a 7 repeat or longer were analyzed as one group.

Genotyping of the VNTR polymorphism in exon 3 of the MAOA gene.

The VNTR polymorphism in exon 3 of the MAOA gene was amplified using primers F-ACAGCCTGACCGTGGAGAAG and R-GAACGGACGCTCCATTCGGA. Reactions were performed in a 384-wells format in a total reaction volume of 20 ul containing 10 ng DNA, 0,5 pmol/ul of each primer, 0,2 mM dNTPs, 1,5 mM MgSO4, PCR buffer (1x), PCRx enhancer (1x) and 2,5 U/ul Platinum Taq. PCR cycling consisted of initial denaturation of 2 min at 95° C, and 36 cycles with denaturation of 30 seconds at 95°C, annealing of 30 seconds at 55°C and extension of 1 minute at 68°C. PCR fragments were size-separated on the Labchip GX (Caliper Life sciences) using a HT DNA 5K chip (Caliper Life sciences). The number of MAOA repeats was determined using the size of the PCR-fragments. To assure genotyping accuracy 250 random samples were genotyped for a second time. No discrepancies were found.

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Chapter 6

General discussion

GENERAL DISCUSSION

RATIONALE

Emotional and behavioural problems are highly prevalent during childhood and often persist into adulthood [1]. This chronic course of child psychiatric disorders highlights the need for prevention, early signaling and treatment. Yet, the causes and precursors of child psychiatric disorders are poorly understood. To achieve this objective, we examined the early effect of parental psychiatric symptoms and the effect of HPA-axis related genes on the risk of child emotional problems. To further study the individual vulnerability of children to parental psychiatric problems, we examined the effect of the interaction between parental psychiatric symptoms and HPA-axis related genes during pregnancy and early childhood on child emotional problems. In this chapter, our main findings will be reviewed, methodological challenges and clinical implications will be discussed, and we will reflect on future perspectives.

PARENTAL PSYCHIATRIC SYMPTOMS AND CHILD EMOTIONAL AND BEHAVIOURAL PROBLEMS.

It has long been recognized that psychiatric problems of parents place children at risk for emotional and behavioural problems. Children of depressed mothers display more anxiety disorders, aggression, attention deficits, insecure attachment, poor self-esteem and poor peer relations [2-3]. Also, depression of a parent increases the risk of a child's exposure to marital conflict and poor family functioning [4-5]. Previous studies showed that parental depression and hostility often co-occur. The independent contribution of depressive symptoms and symptoms of hostility of a mother or a father to the risk of child emotional and behavioural problems are unclear. However, insight in the independent contribution of these risk factors could provide important information for prevention and intervention. In chapter 2, we showed that postnatal hostility symptoms of mother and father independently contributed to the risk of child emotional problems. Parental hostility accounted for the association between parental depressive symptoms and child emotional problems. Moreover, the association between parental hostility and child problems was already present during pregnancy. Given the high correlation of hostility symptoms with other psychiatric symptoms, we cannot conclude that the observed effect is specific to hostility symptoms. Also, mothers' experience of family functioning independently contributed to the risk for child emotional problems. Whether this reflects a specific prenatal effect could not be tested, because no postnatal

information about family functioning before the age of 3 years was available. The prenatal effects of depressive symptoms and hostility did not reach significance in the full model, which is arguably overadjusted. Hence we further evaluated the prenatal effects of individual psychological problems (presented in the footnote)[1]. Overall, this study showed that the association between parental depression and child emotional and behavioural problems may be indexed, mediated or confounded by parental hostility. Thus, parental hostility is a risk factor for child emotional and behavioural problems, which could already be observed during pregnancy.

GENES

Robust main effects of candidate genes associated with child emotional and behavioural problems remain scarce. Despite high prior expectations, the genome wide approach has not identified the genes accounting for the heritability estimates of most psychiatric disorders yet. In this thesis, we aimed to identify new genes associated with child emotional and behavioural problems. In chapter 3, we approached the challenge of gene finding in two different ways. In *chapter 3.1*, we generated a new hypothesis based on the results from a GWAS on BMI. This GWAS found an association between BMI and the FTO gene. This FTO gene was found to be associated with increased food intake and eating behaviour. The relation between eating behaviour and other behavioural phenotypes in combination with the high expression of FTO in the brain may indicate that this gene is also associated to child behavioural problems. Therefore, we tested for an association of the FTO minor allele at rs9939609 with food approach, emotional control and symptoms of ADHD in preschool children. These analyses showed that the FTO minor

1 To examine prenatal effects, we performed additional analyses in which we tested a stepwise model for depressive symptoms only and a stepwise model for parental hostility symptoms only. In these analyses, prenatal maternal depressive symptoms were associated with child emotional problems (beta 1.12, 95%CI 1.02;1.23, p = 0.02). Prenatal depressive symptoms of the father were not associated with child emotional problems (beta 1.09, 95%CI 0.99;1.20, p = 0.08). Postnatal depressive symptoms of the mother (beta 1.29, 95%CI 1.18;1.42, p < 0.001) and the father (beta 1.15, 95%CI 1.04;1.25, p = 0.04) were associated with child emotional problems. The results of the stepwise model with hostility symptoms showed that prenatal maternal hostility symptoms were associated with child emotional problems (beta 1.12, 95%CI 1.01;1.22, p = 0.03). Prenatal hostility symptoms of the father were not associated with child emotional problems (beta 1.10, 95%CI 0.99;1.20, p = 0.06). Postnatal hostility symptoms of the mother (beta 1.41, 95%CI 1.28;1.55, p < 0.001) and the father (beta 1.29, 95%CI 1.17;1.41, p < 0.001) were associated with child emotional problems. Thus, if we perform separate analyses for depressive symptoms and hostility, we find significant prenatal and postnatal effects of maternal depression and significant prenatal and postnatal effect of maternal hostility on child emotional problems.

allele was already associated with food approach in preschool children, thus before the association with BMI at age 5 becomes apparent. Also, the minor allele was associated with a decreased risk for symptoms of ADHD and more emotional self-control. This may reflect an effect of advanced development of carriers of the minor allele at rs9939609 suggested previously by Sovio and colleagues [6]. Future studies are needed to further investigate the function of FTO in the brain and its possible pleiotropic effects on child development. This study showed that results from GWAS using standardized biological measures may enhance gene finding in psychiatric genetics by generating hypotheses about possible underlying biological pathways.

In *chapter 3.2*, we sought to find genes related to depressive symptoms by identifying genes associated with cortisol secretion. Several studies have found that depressive patients show hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis[7-9]. It has been suggested that these alterations in cortisol secretion are causally related to the development of depression. The identification of genes related to cortisol secretion may thus enhance the discovery of genes associated with depressive disorders. This study was embedded in the Rotterdam Study. In this population-based cohort of the elderly, participants collected saliva samples at home. In these samples, the total cortisol concentration during the day was measured and expressed as the area under the curve (cortisol$_{AUC}$). First, a candidate gene study of cortisol$_{AUC}$ was performed. Based on an expert´s opinions, we selected 33 candidate genes known to be involved in central regulation of the HPA-axis, cortisol biosynthesis in the adrenal glands, or the clearance of cortisol from the circulation. After correction for multiple testing by permutations, only common variation in the FKBP5 gene was associated with the cortisol$_{AUC}$, namely with a decrease in cortisol$_{AUC}$. Moreover, carriers of the minor alleles of these SNPs in the FKBP5 gene were at increased risk of clinically relevant depressive symptoms.

Hereafter, a GWAS on cortisol$_{AUC}$ was performed to identify new genes related to cortisol secretion. This GWAS did not identify genome-wide significant SNPs associated with cortisol$_{AUC}$. Since no other cohorts with GWAS data and cortisol data were available to increase the study's power, the top hits were genotyped in Whitehall II Study participants. This replication did not strengthen the results. This may indicate that the initial findings were false-positive due to insufficient power, or that other differences between the study participants introduced heterogeneity in the cortisol measurements.

GENE-ENVIRONMENT INTERACTION DURING PREGNANCY AND EARLY CHILDHOOD

In chapter 4 and chapter 5, we investigated the individual genetic vulnerability to the effect of risk factors during pregnancy and early childhood on child emotional and

behavioural development. These early gene-environment effects may at least partly explain why some children of parents with psychiatric symptoms get ill, and others do not. Furthermore, it has been suggested that gene-environment interaction may be one of the various explanations for the issue of missing heritability in psychiatric genetics (ref).

In chapter 4, we evaluated the effect of candidate SNPs located in the GR gene region and the FKBP5 gene region in interaction with child attachment quality on child cortisol reactivity. The results presented in *chapter 4.1* show that variance in the FKBP5 gene and attachment quality are associated with an increased cortisol reactivity evoked by stress. Infants with an insecure-resistant attachment relationship showed increased cortisol reactivity levels. Also, the T allele at rs1360780 was related to cortisol reactivity, indicating that infants with FKBP5-CT and TT genotypes showed increased cortisol reactivity. The significant interaction between FKBP5 rs1360780 and insecure-resistant attachment showed that resistant infants with the FKBP5-TT genotype showed the largest increases in cortisol reactivity. These results add to the growing evidence that FKBP5 is related to differences in GR sensitivity, and to differential activation of the feedback loop of the HPA axis when confronted by a psychological stressor [10].

In *chapter 4.2*, we studied the interaction between HPA-axis candidate SNPs and prenatal maternal psychiatric symptoms on child emotional and behavioural problems. Diathesis-stress models postulate that adversity in fetal life alters the development of neuronal and endocrine responses to stressors and predisposes individuals to disease [11]. Indeed, it has been shown that maternal psychiatric symptoms during pregnancy influence maternal cortisol levels, which in turn have been associated with increased cortisol levels and disrupted behaviour in the offspring [12-13]. In our study, common variation in the GR gene (rs41423247) significantly moderated the association between prenatal maternal psychological symptoms and child emotional and behavioural problems. This prenatal interaction effect was independent of mother's genotype and maternal postnatal psychopathology, and not found for prenatal psychological symptoms of the father. In addition to an effect on child behaviour, the minor allele at rs41423247 and prenatal maternal psychological symptoms interacted to influence child cortisol reactivity, resulting in decreased cortisol levels after exposure to stress. In summary, we found evidence for prenatal gene-environment interaction affecting HPA-axis functioning and the risk of child emotional and behavioural problems.

During pregnancy, the HPA-axis of the fetus is programmed according to the circumstances in the intra-uterine environment in anticipation of life outside the womb. In our studies, we did not have information about maternal cortisol levels, but there is mounting evidence from previous studies showing that mothers with psychiatric problems during pregnancy have increased cortisol levels [13]. Despite the barrier function of the placenta, maternal cortisol will reach the fetus. In response, the fetal HPA-axis develops under the influence of increased cortisol levels. This fetal anticipation is highly effective

if indeed the circumstances outside the womb match the stress experienced during fetal development. However, the HPA-axis of these children may be too reactive to the environment, also in the absence of true danger, which may result in chronic activation of the HPA-axis and related psychopathology.

In *chapter 4.1*, the interaction between FKBP5 and insecure attachment were associated with increased stress reactivity after the SSP. Hence, the attachment relationship was assessed at the same time as child cortisol reactivity and thus measures the acute stress response. The prenatal GxE presented in *chapter 4.2* may represent a more chronic effect of prenatal maternal psychiatric problems on the HPA-axis, since this GxE was associated with decreased cortisol reactivity at 3 years.

In chapter 5, we focused on moderation by 5-HTTLPR on the relation between maternal psychiatric symptoms and child development. Brown and Harris suggested that 5-HTTLPR may have an early effect on neurodevelopment, which may increase the risk for psychopathology over the lifespan [14]. In our study presented in *chapter 5.1*, 6 month-old infants with the short allele of the 5-HTTLPR were more likely to be negatively emotional when mother reported anxiety symptoms during pregnancy than long allele carriers. This early moderation of 5-HTTLPR was further studied in *chapter 5.2*. In this chapter, we showed that 5-HTTLPR moderates the effect of prenatal maternal anxiety symptoms on child emotional problems and child emotion processing. Independent of this prenatal effect, 5-HTTLPR also interacted with postnatal maternal anxiety symptoms to influence the risk for child emotional problems. These results provide initial evidence for early moderation by 5-HTTLPR on the effect of maternal psychiatric symptoms on child emotional development.

METHODOLOGICAL CHALLENGES

In the chapters 2 to 5, we discussed the methodological considerations for each study and address the possible influence of selection bias, information bias and confounding on our results. In this section, we will only discuss the challenges related to cortisol research and gene-environment interaction analyses but with more detail than possible in typical peer-reviewed manuscripts.

Cortisol

Several investigators have argued that cortisol is a suitable biomarker of anxious or depressed disorders [7, 9]. We will interpret such findings as suggesting that cortisol is a biological correlate of some psychiatric disorders. Still, the challenge is that cortisol is the end product of a complex hormonal axis involved in many other systems than the psychological stress response. The diurnal rhythm of cortisol secretion follows the cycle

of activity and rest. It prepares the body for demands, regulates energy metabolism, and aims at homeostasis. Further, inflammatory processes will influence cortisol secretion [15]. In addition to this daily rhythm, cortisol is indeed released in stressful circumstances to make up the coping or adaptive response. Also, the secretion of cortisol is very adaptive and characterized by large intra- and inter-individual variability [16]. For instance, cortisol secretion varies between sexes and changes with age. Life style factors such as physical activity, alcohol consumption, coffee intake, and smoking may further influence cortisol levels. Hence, optimal levels of cortisol that could be used as reference for making clinical decisions cannot be defined [17]. Therefore, cortisol reactivity in response to stress may be more informative, because this will give information about the response of the HPA-axis under stress compared to baseline cortisol levels. Instead of exposure to stress, the HPA-axis responsivity can be chemically challenged with the combined dex/CRH test [18]. In this suppression test, dexamethasone is orally administered and expected to reduce cortisol secretion. Elevated plasma cortisol responses have been found in patients with an acute major depression disorder. This escape from the suppressive effect of dexamethasone has been explained by impaired GR signaling in these patients. Although the use of such standard stimuli may yield a more narrow phenotype reflecting HPA-axis functioning, other psychiatric disorders and medical conditions are also associated with an elevated response. Importantly, the required intake of dexamethasone in combination with several blood collections make it less feasible for large population-based studies, especially when these involve children.

There is evidence to suggest that it is mainly the cortisol awakening response (CAR) that is under genetic influence [16]. The CAR is present within the first 45 minutes after awakening. We performed a GWAS on the total cortisol secretion during the day, because it is difficult to obtain reliable assessment of the CAR in large scale population-based studies. People are required to sample saliva at awakening, and then again at 15 and 30 minutes after awakening. Especially in a study of elderly participants, the compliance to this type of assessment is low. Therefore, we used the total cortisol secretion during the day, which also included the CAR.

Gene-environment interaction studies

Due to the high rate of non-replication of most initial findings, the validity of GxE interaction studies is under debate. Especially during pregnancy, the empirical evidence remained scarce. First, we will discuss the issue of non-replication in GxE studies. Second, the impact of GxE on the phenotype is discussed. Third, we will touch upon the differential susceptibility framework.

The most renowned GxE interaction is probably the interaction between 5-HTTLPR and stressful life events on risk of depression reported by Caspi and colleagues [19]. In this study, the risk of a major depression disorder increased as the number of stressful life events increased, but only in s-allele carriers. Following this initial report, three sub-

sequent meta-analyses tried to answer the question whether this GxE truly exists. Based on the results of their meta-analyses, Munafo and colleagues and Risch and colleagues concluded that the initial report is most likely a chance finding [20-21]. These authors mainly criticize the interaction analyses in the absence of a genetic main effect. The absence of a genetic main effect on depression would require a reversal in the genetic effect in the absence of life events, which they did not observe. They also argue that replication attempts are difficult due to heterogeneity between studies in designs, measurements and methods. Opposed to these two meta-analyses, Karg and colleagues found strong evidence for the interaction between 5-HTTLPR and life events on depression [22]. They showed that stressor type and stress assessment method greatly influence the study outcome. Studies vary in the definition of life events. The GxE was stronger for chronic stressors, and stronger among studies using observational measurements. This seems to indicate that we should not debate the concept of GxE, but we should discuss how the analyses can be improved. Part of the inconsistency may be explained by the fact that people differ greatly in their ability to cope with stressful life events. In chapter 5.2, the interaction between 5-HTTLPR and maternal chronic difficulties during pregnancy on child emotional problems was no longer significant after adjustment for the interaction between 5-HTTLPR and prenatal anxiety. This may indicate that merely the presence of chronic difficulties does not imply increased stress or psychopathology. Hence, GxE studies will possibly benefit most from a more careful selection and measurement of the environment.

In response to the inconsistent findings, several papers have been published suggesting how to improve the quality of GxE studies in psychiatry. Some even suggested strict guidelines [23-25] or underscore the need for high quality replication efforts [26]. Caspi and colleagues stress upon the importance of construct validity over statistically replicated GxE findings. Construct validation searches for associations that are confirmed despite variation in sample characteristics, phenotype measurements or environmental exposure [27]. Our findings in chapter 5 further strengthen the evidence for the 5-HTT stress sensitivity hypothesis.

Second, the impact of most GxE on the phenotype is small. In GxE studies, the effect of a single measure of molecular genetic variation in interaction with the environment on the phenotype is examined. The prior probability is usually low, because it concerns a single base pair change. In our studies, about 1%, of the phenotypic variance is due to gene-environment interaction. Albeit a small proportion of the explained variance, this magnitude of an effect is what can be expected from a GxE with one single SNP in the general population. Most families in our study are fairly healthy and only a small proportion of parents and children show psychiatric symptoms. The effect estimates may be larger in clinical samples.

Third, we studied GxE within the framework of a diathesis-stress model. In addition to this classic model, the differential susceptibility model assumes that children differ

in sensitivity to the environment according to their genetic make-up, temperament or biological sensitivity in a "for better or for worse" manner [28]. 5-HTTLPR may be such a plasticity gene. Children with the short allele of the 5-HTTLPR may do worse in an adverse environment, but may benefit from a positive environment [29]. If the direction of the effect of a common variant indeed depends on the quality of the environment, this could also explain the small effect estimates observed for the disadvantageous environment only. In this thesis, we did not find strong evidence for differential susceptibility, except for the interaction of 5-HTTLPR with prenatal maternal anxiety on observed child emotion-matching accuracy. In these analyses, s/s carriers with mothers reporting low prenatal anxiety, the emotion-matching accuracy was better compared to l/s and l/l carriers. Whereas the s/s carriers with mothers reporting high prenatal anxiety were least accurate on emotion-matching. Future studies should consider the possibility of differential susceptibility and also study genetic effects under advantageous circumstances.

CLINICAL IMPLICATIONS

Clearly, it will take time before these fundamental findings, especially on gene-environment interaction, will be of direct use for the clinical practice. Hence, direct implication of the presented studies for the clinical practice are limited. Here, we elaborate on two possible implications.

First, we found a comparatively large impact of maternal and paternal hostility on child emotional and behavioural problems, especially if contrasted to the effect of parental depression or chronic parental difficulties. Moreover, the effect of parental hostility could already be detected during pregnancy. Arguably, it is important to better consider parental hostility as a risk factor for child emotional and behavioural problems.

Second, we reported several independent associations of genetic variation in the FKBP5 gene with diurnal cortisol secretion, cortisol reactivity evoked by stress, depressive symptoms in the elderly, and child attachment quality. With these findings, we add to the mounting evidence that FKBP5 plays an important role in HPA-axis regulation and depression. Clinical and preclinical data support the assumption that normalization of impaired corticosteroid receptor signaling is the final common pathway of antidepressants with different pharmacological profiles. It was recently suggested that the FKBP5 gene may be a new target for the development of antidepressant therapies [30]. Thus, our fundamental findings seem to add to the construct validity of the role of FKBP5 in depression, and may enhance the development of new antidepressant therapies.

FUTURE PERSPECTIVES

In light of the ongoing search for the missing heritability, future studies should further investigate the role of rare SNPs, copy number variations (CNVs), epistasis and epigenetics in the etiology of child emotional and behavioural problems. In relation to HPA-axis regulation, epigenetics merit further attention. Future gene-environment interaction studies may benefit from a more careful assessment of the environment. These two suggestions are further discussed in this section.

Epigenetic processes represent a mechanism by which the environment affects the function of a gene. Variation in maternal postnatal care in rats has been associated with increased methylation in the GR promoter in the hippocampus and HPA-axis response to stress [31]. Also, maternal depressed mood during pregnancy has been associated with increased methylation of the *GR* promoter region in neonates, which is turn predicted increased HPA-axis reactivity in these neonates at 3 months [32]. It would be very interesting to examine the long term effect of early methylation of the GR gene region on child HPA-axis regulation and emotional development.

Next to the promising findings in epigenetics, there is still a lot to gain in gene-environment interaction studies. Non-replication should be reduced by a more careful selection and measurement of the environment. The impact of life events and chronic difficulties likely depends on other factors such as coping styles and support in the environment. So, individual coping mechanisms will affect whether these difficulties will lead to stress and anxiety, but are currently not included in GxE studies. Differential susceptibility also shed another light on GxE studies. Non-replication may be the result of opposite genetic effect depending on the quality of the environment. Hence, future studies should not only invest in the assessment of determinants of a negative environment, but also study genetic effects on positive environments.

To conclude, this thesis focused on nurture versus nature and the interplay. Our studies provided important evidence for gene-environment interaction during pregnancy and early childhood. Genes related to HPA-axis functioning interact with the early environment to influence the risk of child emotional and behavioural problems in preschoolers. In this chapter, we concluded that the concept of GxE should not be the focus of the debate. Rather, the focus should be on the study design including observational assessments, repeated measurements and multiple informants. Importantly, future GxE studies should better assess the environmental risk factors. Only if we measure the environment in more detail, with more precision and over time, will we understand how it influences and interacts with biology. The interplay with biology determines the eventual effect of the environment on the phenotype. Nurture versus nature: there and back again [33].

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Chapter 7

Summary/ Samenvatting

SUMMARY

Childhood psychiatric disorders are common, show a high comorbidity and are associated with a long-term vulnerability for mental health problems, which underscores the importance of a better understanding of their etiology. Psychiatric symptoms of the parents place children at risk for the development of emotional and behavioural problems. Previous studies showed that parental depression and hostility often co-occur. The independent contributions of depressive symptoms and symptoms of hostility of a mother or a father to the risk of child emotional and behavioural problems are unclear. Twin studies reported moderate to high heritability estimates for psychiatric disorders. The actual genes accounting for these estimates remain to be determined. In light of the inherent complexity of psychiatric disorders, it seems likely that these disorders are caused by small effects of many genes in interaction with other genes and in interaction with the environment. As described in Chapter 1, the aim of this thesis was to study the effect of common genetic variation, parental psychiatric symptoms and the effect of their interaction during pregnancy and early childhood on the risk of emotional and behavioural problems in preschool children.

The studies in this thesis were embedded in The Generation R Study, a large prospective population-based cohort study from fetal life onwards in the city of Rotterdam, the Netherlands. It was designed to identify early biological and environmental determinants of growth, development and health in fetal life and childhood. Between 2002 and 2006, 8,880 pregnant women enrolled in the prenatal part of the study. In total, 7,893 children participated in the postnatal phase of the Generation R Study. The study described in *Chapter 3.2* was embedded in the Rotterdam Study, a cohort of elderly people in the city of Rotterdam, the Netherlands.

The first part of this thesis focused on the impact of prenatal and postnatal psychiatric symptoms and family function of both mothers and fathers on the risk for child emotional and behavioural problems. In Chapter 2, we showed that postnatal hostility symptoms of mother and father independently contributed to the risk of child emotional problems. Parental hostility accounted for the association between parental depressive symptoms and child emotional problems. These findings suggest that the association between parental depression and child emotional and behavioural problems may be indexed, mediated or confounded by parental hostility. These findings also showed that parental hostility is a strong risk factor for child emotional and behavioural problems, which could already be observed during pregnancy.

The second part of this thesis focused on genetic main effects on child emotional and behavioural problems. In *Chapter 3.1*, we examined the association between a candidate SNP in the FTO gene and child (eating) behaviour. Previously, this FTO gene was found to be associated with increased BMI, increased food intake and eating behaviour. Given the

relation between eating behaviour and other behavioural phenotypes in combination with the high expression of FTO in the brain, we hypothesized that this gene may also be associated with child behavioural problems. We showed that the FTO minor allele was already associated with food approach in preschool children, thus before the association with BMI at age 5 becomes apparent. The minor allele was also associated with a decreased risk for symptoms of ADHD and more emotional self-control. This may reflect advanced development of carriers of the minor allele at rs9939609. It may also suggest that the FTO gene has pleiotropic effects on child development.

In *Chapter 3.2*, we sought to identify new genes related to both cortisol secretion and depression using a GWAS approach and a candidate gene approach.

Our candidate gene study of cortisol$_{AUC}$ showed that common variation in the FKBP5 gene was associated with a decrease in cortisol$_{AUC}$. Carriers of the minor alleles of SNPs in the FKBP5 gene were also at increased risk of clinically relevant depressive symptoms. These findings support the relation between HPA-axis regulation and depressive symptoms. To identify new genes related to HPA-axis functioning, we performed a GWAS on cortisol$_{AUC}$. This GWAS did not identify genome-wide significant SNPs associated with cortisol$_{AUC}$, which may be due to insufficient power.

The negative replication results may indicate that the initial findings were indeed false-positive, but could also be the result of heterogeneity in the cortisol measurements among our study sample and the replication sample.

The third part of this thesis focused on the effect of the interaction between HPA-axis related genes and parental psychiatric symptoms on child emotional and behavioural problems. In *Chapter 4.1*, we evaluated the effect of candidate SNPs located in the GR gene region and the FKBP5 gene region in interaction with child attachment quality on child cortisol reactivity. This study showed that variance in the FKBP5 gene and attachment quality are associated with an increased cortisol reactivity evoked by stress. Resistant infants with the FKBP5-TT genotype showed the largest increases in cortisol reactivity. In *Chapter 4.2*, we hypothesized that children carrying the minor alleles of SNPs located in the GR gene region and the FKBP5 gene region would be more vulnerable to the effect of maternal psychiatric symptoms during pregnancy, resulting in an increased risk of emotional and behavioural problems. In this study, common variation in the GR gene at rs41423247 (BclI1) significantly moderated the association between prenatal maternal psychological symptoms and child emotional and behavioural problems. This prenatal interaction effect was independent of mother's genotype and maternal postnatal psychopathology, and not found for prenatal psychological symptoms of the father. In addition to an effect on child behaviour, the minor allele at rs41423247 and prenatal maternal psychological symptoms interacted to influence child cortisol reactivity, resulting in decreased cortisol levels after exposure to stress.

In Chapter 5, we hypothesized that 5-HTTLPR interacts with prenatal and postnatal maternal anxiety symptoms to influence child emotional development. In *Chapter 5.1*, we showed that 6 month-old infants with the short allele of the 5-HTTLPR were more likely to be negatively emotional when mother reported anxiety symptoms during pregnancy than long allele carriers. This early moderation of 5-HTTLPR was further studied in *Chapter 5.2*. In this chapter, we showed that 5-HTTLPR moderates the effect of prenatal maternal anxiety symptoms on child emotional problems and child emotion processing. Independent of this prenatal effect, 5-HTTLPR also interacted with postnatal maternal anxiety symptoms to influence the risk for child emotional problems. These results provide initial evidence for early moderation by 5-HTTLPR on the effect of maternal psychiatric symptoms on child emotional development

In Chapter 6, the main findings of these studies were reviewed, methodological considerations and clinical implications were discussed and we reflected on future perspectives. In short, we showed that HPA-axis related genes may moderate the effect of prenatal and postnatal maternal psychological symptoms on child emotional and behavioural problems. During pregnancy, the HPA-axis of the fetus is programmed according to the circumstances in the intra-uterine environment in anticipation of life outside the womb. Mothers with psychiatric problems during pregnancy have increased cortisol levels. Despite the barrier function of the placenta, maternal cortisol will reach the fetus. In response, the fetal HPA-axis develops under the influence of increased cortisol levels. This fetal anticipation is highly effective if indeed the circumstances outside the womb match the stress experienced during fetal development. However, the HPA-axis of these children may be too reactive to the environment, also in the absence of true danger, which may result in chronic activation of the HPA-axis and related psychopathology. These early gene-environment effects may at least partly explain why some children of parents with psychiatric symptoms get ill, and others do not. Furthermore, this gene-environment interaction may partly account for the missing heritability in psychiatric genetics.

Several investigators have argued that cortisol is a suitable biomarker of anxious or depressed disorders. The challenge is, however, that cortisol is the end product of a complex hormonal axis involved in many other systems than the psychological stress response. Therefore, optimal levels of cortisol that could be used as reference for making clinical decisions cannot be defined. Thus, it will take time before these fundamental findings will be of direct use for the clinical practice. Our findings do suggest that it is important to better consider parental hostility as a risk factor for child emotional and behavioural problems next to depression of parents. Second, our findings support the important role of FKBP5 in depression, and may enhance the development of new antidepressant therapies.

Epigenetic processes represent a mechanism by which the environment affects the function of a gene. It would be very interesting to examine the long term effect of early methylation of the GR gene region on child HPA-axis regulation and emotional development.

Due to the high rate of non-replication of most initial findings, the validity of GxE interaction studies is under debate. Especially during pregnancy, the empirical evidence remained scarce. We concluded that the concept of GxE should not be the focus of the debate. Rather, the focus should be on the study design including observational assessments, repeated measurements and multiple informants. Importantly, future GxE studies should better assess the environmental risk factors. Only if we measure the environment in more detail, with more precision and over time, will we understand how it influences and interacts with biology. Ultimately, this insight will answer to the question why some children get ill and others do not.

SAMENVATTING

Psychiatrische stoornissen op de kinderleeftijd komen vaak voor, kenmerken zich door een hoge comorbiditeit en zijn geassocieerd met een verhoogde kwetsbaarheid voor psychiatrische aandoeningen op de lange termijn. Daarom is het van belang om de oorzaken van deze stoornissen beter te begrijpen.

Kinderen van ouders met psychiatrische problemen hebben een verhoogd risico op emotionele en gedragsproblemen. Depressie en vijandigheid van ouders komen vaak samen voor. Het is onbekend wat de afzonderlijke bijdragen zijn van depressie en vijandigheid van ouders aan het risico op psychiatrische symptomen bij hun kinderen. Uit onderzoek onder tweelingen is gebleken dat de meeste psychiatrische stoornissen in hoge mate erfelijk zijn. De genen die hier verantwoordelijk voor zijn moeten echter nog gevonden worden. Het is waarschijnlijk dat complexe aandoeningen zoals psychiatrische ziektebeelden worden veroorzaakt door kleine effect van meerdere genen in interactie met andere genen en omgevingsfactoren. In dit proefschrift zijn studies gebundeld waarin we het effect hebben onderzocht van genen, psychiatrische symptomen van de ouders, en hun interactie op het risico van emotionele en gedragsproblemen bij jonge kinderen.

De studies in dit proefschrift werden verricht binnen de Generation R Study, een groot bevolkingsonderzoek in Rotterdam waarin kinderen en hun ouders worden gevolgd vanaf de zwangerschap. Het doel van de Generation R Study is om vroege biologische en omgevingsdeterminanten te identificeren die van invloed zijn op de groei, ontwikkeling en gezondheid van kinderen. Tussen 2002 en 2006 werden 8880 zwangere vrouwen geïncludeerd. In totaal namen 7893 kinderen deel aan het postnatale deel van de Generation R Study. De studie beschreven in *hoofdstuk 3.2* werd verricht in the Rotterdam Study, een cohort van ouderen in Rotterdam.

In het eerste deel van dit proefschrift onderzochten we de relatie tussen psychiatrische symptomen van ouders en het functioneren van het gezin tijdens en na de zwangerschap op het risico op emotionele en gedragsproblemen van jonge kinderen. In hoofdstuk 2.1 laten we zien dat vijandigheid van vaders en moeders na de zwangerschap onafhankelijk bijdraagt aan problemen bij kinderen. Bovendien verklaarde vijandigheid van de ouders grotendeels het effect van depressie van ouders op problemen bij kinderen. Dit suggereert dat de vijandigheid van ouders een confounder en/of een mediator is in de relatie tussen depressie van ouders en problemen bij kinderen. Deze bevindingen laten ook zien dat vijandigheid van de ouders een sterke risicofactor is voor emotionele en gedragsproblemen bij kinderen, wat al geobserveerd kan worden tijdens de zwangerschap.

In het tweede deel bestudeerden we genetische hoofdeffecten op emotionele en gedragsproblemen bij kinderen. In *hoofdstuk 3.1* beschrijven we de relatie tussen een

polymorfisme (rs9939609) in het FTO gen en (eet)gedrag van kinderen. Dit FTO gen werd eerder geassocieerd met een verhoogd risico op een hoog BMI, grotere hoeveelheden eten en eetgedrag. Aangezien eetgedrag is gerelateerd aan andere fenotypes van gedrag en dit FTO gen tot hoge expressie komt in de hersenen, hebben wij getoetst of de risico-variant van het FTO gen ook direct is geassocieerd me gedragsproblemen in kinderen. Onze studie laat zien dat het polymorfisme (rs9939609) is gerelateerd aan eetgedrag in jonge kinderen, al voordat de relatie met dit gen en BMI duidelijk is. Daarnaast is dit polymorfisme geassocieerd met een verlaagde kans op symptomen van ADHD en meer emotionele controle. Dit kan betekenen dat dragers van het polymorfisme zich sneller ontwikkelen. Het kan ook duiden op een pleiotropisch effect van het FTO gen tijdens de ontwikkeling van kinderen.

In de studie beschreven in *hoofdstuk 3.2* was het doel om nieuwe genen te identifceren die gerelateerd zijn aan cortisol secretie en depressie. Onze kandidaat gen studie laat zien dat het FKBP5 gen is geassocieerd met een afgenomen cortisol secretie gedurende de dag. Dragers van polymorfismen in het FKBP5 gen liepen ook een verhoogd risico op klinisch relevante depressieve symptomen. Deze bevindingen ondersteunen de relatie tussen het functioneren van de HPA-as en depressie symptomen. Naast de kandidaat gen studie hebben we een genoom-brede studie uitgevoerd op cortisol secretie gedurende de dag. We hebben geen significante associaties gevonden tussen genetische polymorfismen en cortisol, mogelijk door onvoldoende statistische power. De negatieve replicatie resultaten duiden mogelijk op fout-positieve bevindingen in onze studie, of zijn mogelijk het resultaat van te grote verschillen in de cortisol metingen in onze studie en het replicatie cohort.

In het derde deel van dit proefschrift hebben we ons gericht op de interactie tussen HPA-as gerelateerde genen en psychiatrische symptomen van de ouders en het effect hiervan op problemen bij kinderen. In *hoofdstuk 4.1* beschrijven we het effect van polymorfismen in het Glucocorticoid Receptor gen en het FKBP5 gen in interactie met de kwaliteit van de gehechtheidsrelatie op cortisol reactiviteit in kinderen. In deze studie vonden we een gezamenlijk effect van een specifieke variant van het FKBP5 gen (rs1360780) en onveilig-ambivalente gehechtheid. Onveilig-ambivalent gehechte kinderen die de risico-variant van rs1360780 droegen hadden een verhoogd risico op een toename in cortisol waardes na een stressvolle situatie. In *hoofdstuk 4.2*, hebben wij de hypothese getest of kinderen met risico-varianten van verschillende SNPs in het GR gen en het FKBP5 gen die daarbij moeders hadden met psychiatrische symptomen tijdens de zwangerschap een verhoogd risico hadden op emotionele en gedragsproblemen op de kinderleeftijd. Wij vonden dat dragers van de risico-variant op rs41423247 (BCLI1) van moeders met psychiatrische problemen tijdens de zwangerschap een verhoogd risico hadden op problemen. Deze kinderen hadden ook een verhoogd risico op toename van cortisolwaardes na een stressvolle situatie. Dit effect werd niet verder verklaard door de

genetische variant van de moeder of door psychiatrische problemen van de moeder na de zwangerschap. We vonden dit interactie effect niet met psychiatrische problemen van de vader wat suggereert dat dit een effect is wat in de baarmoeder plaatsvindt. Deze bevindingen onderschrijven het 'dubbel-risico-model', waarbij meerdere risicofactoren bijdragen aan een toegenomen kans op negatieve uitkomsten.

In hoofdstuk 5 hebben we het effect van interactie tussen de korte variant in de promoter regio van het serotinine transporter gen (5-HTTLPR) met angst klachten van de moeder tijdens en na de zwangerschap onderzocht. Op de leeftijd van 6 maanden lieten zuigelingen met de korte variant en moeders met prenatale angst klachten meer negatieve emoties zien dan kinderen met de lange variant (*hoofdstuk 5.1*). In *hoofdstuk 5.2* hebben wij laten zien dat 5-HTTLPR de relatie tussen prenatale en postnatale angst klachten beïnvloedt. Deze interactie heeft effect op het verwerken van emoties en op het risico op emotionale problemen op de leeftijd van 5 jaar. Deze resultaten laten zien dat 5-HTTLPR al vroeg in de ontwikkeling het effect van de omgeving beïnvloedt en de kans op emotionele problemen bij jonge kinderen verhoogd.

In hoofdstuk 6 bespreken we de voornaamste bevindingen, methodologische zaken, klinische implicaties en suggesties voor vervolg onderzoek. De studies beschreven in dit proefschrift laten zien dat genen betrokken bij HPA-as regulatie het effect van psychiatrische symptomen van moeders tijdens en na de zwangerschap beïnvloeden. Tijdens de zwangerschap wordt de foetus voorbereid en geprogrammeerd op de omstandigheden buiten de baarmoeder. Moeders met psychiatrische problemen hebben verhoogde cortisol waardes die ook de foetus bereiken. Onder deze condities met verhoogde cortisol waardes wordt de HPA-as van de foetus ontwikkeld. Dit mechanisme is effectief als de omstandigheden buiten de baarmoeder inderdaad stressvol zijn. Echter, de HPA-as van deze kinderen is mogelijk te reactief, ook als er geen gevaar dreigt. Dit zou kunnen leiden tot een chronische activatie van de HPA-as en het stress system, wat is gerelateerd aan psychiatrische problemen. Deze vroege gen-omgevings interactie zou deels kunnen verklaren waarom sommige kinderen van moeders met psychiatrische symptomen wel ziek worden en anderen niet. Op basis van genetische eigenschappen lijkt er een verschil te bestaan in kwetsbaarheid voor het effect van psychiatrische symptomen van de moeder.

Er is verondersteld dat cortisol een geschikte biomarker is voor angst en depressie. De uitdaging is echter dat cortisol het eindproduct is van een complex hormonaal systeem. Dat maakt het heel moeilijk om optimale waardes te definiëren die als klinisch afkappunt kunnen worden gebruikt. Daarom zijn onze fundamentele bevindingen nog niet direct van nut voor de klinische praktijk. Onze resultaten laten wel zien dat vijandigheid van ouders een belangrijke voorspeller is van problemen bij kinderen, naast het effect van depressie van ouders. Ten tweede laten onze resultaten zien dat het FKBP5 gen een

belangrijke rol spelt bij depressie, en mogelijk een rol kan spelen bij de ontwikkeling van nieuwe antidepressiva.

Een van de manieren waarop de omgeving het functioneren van een gen beïnvloedt is via epigenetische processen. Vervolg onderzoek zou zich moeten richten op het effect van vroege methylering van het GR gen op de regulatie van de HPA-as en de emotionele ontwikkeling van kinderen op de lange termijn.

Vanwege de hoge mate van non-replicatie van GxE studies staat de validiteit van deze studies ter discussie. Met name tijdens de zwangerschap is er nog weinig bewijs voor GxE effecten. Wij concluderen dat niet zozeer het concept van gen-omgevingsinteractie het onderwerp van de discussie zou moeten zijn, maar eerder het ontwerp en de manier van het uitvoeren van deze studies. De nadruk zou meer moeten liggen op het includeren van observationele maten, gebruik van herhaalde metingen en meerdere informanten. Daarbij is het van belang dat er meer aandacht komt voor het meten en in kaart brengen van de omgeving. Dit kan met meer precisie en over een langere periode, zodat we beter kunnen begrijpen hoe en wanneer de interactie met genen daadwerkelijk tot stand komt. Uiteindelijk zal dit bijdragen aan verder inzicht in de individuele kwetsbaarheid van kinderen voor de omgeving tijdens en na de zwangerschap.

Chapter 8

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About the author
PhD portfolio
After word

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ABOUT THE AUTHOR

Fleur Pieternel Velders was born in the city of Groningen, the Netherlands, on March 9th 1981. She passed secondary school in 1999 at RSG Magister Alvinus in Sneek. In the same year she started medical school at the Erasmus Medical Center of Rotterdam. During medical school, she was an active member of the students' society "Het Rotterdamsch Studenten Gezelschap" and participated in several committees. In 2002, she examined the practical implications of the Roll Back Malaria project of the World Health Organization in Mali, Western-Africa. In 2004, she graduated on `Fever without source` under the supervision of Prof. Dr. H.A. Moll. As part of her internships, she worked as a trainee at the Department of Pediatrics and the Department of Endocrinology at the University of Otago in Wellington, New Zealand from January until April 2006.

After obtaining her medical degree in 2006, she started her first job as MD at the Department of Child and Adolescent Psychiatry of the Erasmus Medical Center. In February 2007, she started the work described in this thesis under supervision of Prof. Dr. H. Tiemeier, Prof. Dr. F.C. Verhulst and Drs. G. Dieleman. As part of this PhD-project, she obtained a Master of Science degree in Health Sciences (Genetic Epidemiology) in August 2010.

In March 2012, she started her residency in psychiatry at Pro Persona Arnhem under supervision of Mr. M. Braakman and Prof. Dr. RJ. van der Gaag. When she is a child and adolescent psychiatrist, she would like to combine patient care with scientific research.

Fleur is married to Sebastiaan Berendse. In 2008 and 2010, their two daughters Elin and Moira were born.

PHD PORTFOLIO SUMMARY

Summary of PhD training and teaching activities

Name PhD student: Fleur Velders	PhD period: 2007-2012
Erasmus MC Department: Child and Adolescent Psychiatry	Promotor(s): Prof. Dr. F. C. Verhulst Prof. Dr. H. Tiemeier
Research School: NIHES	

1. PhD training

	Year	Workload (ECTS)
General academic skills		
- Biomedical English Writing and Communication	2009	4.0
Research skills		
- Principles of Research in Medicine and Epidemiology	2007	0.7
- Clinical trials	2007	0.7
- Topics in Evidence-based Medicine	2007	0.7
- Study Design	2007	4.3
- Classical Methods for Data-analysis	2007	5.7
- Conceptual foundation of Epidemiologic Study Design	2008	0.7
- Cohort studies	2008	0.7
- Decision-making in Medicine	2008	0.7
- History of Epidemiological Ideas	2008	1.7
- Repeated Measurements in Clinical Studies	2008	1.4
- Missing Values in Clinical Research	2008	0.7
In-depth courses (e.g. Research school, Medical Training)		
NIHES Master of Genetic Epidemiology		
- Genetic Epidemiology of Complex Diseases	2007	1.4
- Introduction to Genomics and Bioinformatics	2007	0.7
- Principles of Genetic Epidemiology	2007	0.7
- Genetic-epidemiology Research Methods	2007	5.7
- SNPs and Human Diseases	2007	1.8
- Advances in Population-based Studies of Complex Genetic Disorders	2009	1.4
- Genetic Linkage Analysis: Model-based Analyis	2009	1.4
- Genetic Linkage Analysis: Model-free Analyis	2009	1.4
- Advances in the Statistical Analysis of Pedigree Data	2009	1.1
- Mendelian Randomization	2010	0.6
Presentations		
- World Congress of Psychiatric Genetics, San Diego, oral presentation	2009	0.3
- Department of Psychiatry, Utrecht Medical Center, the Netherlands, oral presentation	2010	0.3
- International Consortium (EAGLE) meeting, Oslo, oral presentation	2010	0.7
- World Congress of Psychiatric Genetics, Athens, poster presentation (presented by G. Dieleman)	2010	0.5
- Publieksdag Jubileum Congres NVvP, Amsterdam, oral presentation	2011	0.6
- International Consortium (EAGLE) meeting, London, oral presentation	2012	1.0
- International Expert meeting Gene-Environment Interaction, Utrecht, oral presentation	2012	0.5
- NVvP Spring Conference, Maastricht, Symposium Genetics in Child and Adolescent Psychiatry, oral presentation	2012	0.5

Seminars and workshops		
- "The power of early experiences: On brain plasticity, sensitive periods and biobehavioral recovery from early trauma." Faculty of Social Sciences, Leiden University.	2009	0.1
- "Towards an integrative theory of ADHD: Current state of the art and future perspectives."	2009	0.1
- 6th Annual CMSB (Centre for Medical Systems Biology) Symposium.	2009	0.1
- International Consortium (EAGLE) Meeting, London	2009	0.4
- Congress of the Netherlands association of Psychiatry (NVvP), Maastricht	2010	0.2
Other		
- Deputy PhD students in the Generation R Management Team	2007-2008	5.0
- Logistic Manager of the EAGLE working group Cognition and Behaviour	2009-2011	8.0
2. Teaching activities		
- Supervising medical students about observation skills and childhood psychiatric disorders (thema 3.2) Erasmus Medical University, Rotterdam, the Netherlands	2007/2008	1.0
- Lecturing students, Department of Psychology, Erasmus university Rotterdam	2007/2008	0.3
- Supervising Master's thesis "*FTO* and behavioural problems" Jolanda de Wit, medical student, Erasmus Medical Center.	2011	10.0
1 ECTS (European Credit Transfer System)is equal to a workload of 28 hrs.		