Time after time: biological factors in the course of recurrent depression
Lok, A.

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Time after Time; biological factors in the course of recurrent depression

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Anja Lok
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Promotores:
  Prof. dr. A.H. Schene
  Prof. dr. C.L.H. Bockting

Copromotores:
  Dr. M.W.J. Koeter
  Dr. J. Assies

Overige leden:
  Prof. dr. B.W.J.H. Penninx
  Prof. dr. M. Olff
  Prof. dr. R.C. van der Mast
  Prof. dr. W. van den Brink
  Prof. dr. E.M. Derks

Faculteit der Geneeskunde
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Chapter 1
General Introduction
General Introduction

1.1 Introduction
1.1.1 Introduction and outline of thesis

Major Depressive Disorder (MDD) is a heterogeneous disorder with a highly variable and recurrent course, an inconsistent response to treatment, and is thus far without an established aetiology. This thesis presents some biological approaches associated with and/or predicting the course of recurrent depression.

The structure of this introductory chapter is as follows: first an introduction will be given on the recurrent type of MDD. This is followed by its relationship to cardiovascular risk. Some pathophysiological mechanisms, which possibly influence the course of MDD, will then be introduced. Thereafter, an overview of the DELTA-study, which forms the basis of most of the data used in this thesis, will be presented in association with a timeline figure. Lastly, the general aims and an outline of the succeeding chapters of the thesis will be given.

Recurrent depression

MDD is one of the most common forms of mental illness and affects approximately one in six men and one in four women over a lifetime 1. More recently, Bromet et al. 2 reported that the occurrence of depressive episodes over a 12-month period was found to be 5.9% in low-middle income countries compared to 5.5% in high-income countries. MDD is also highly recurrent*, as at least 50% of those who recover from an initial episode of depression will suffer from experiencing one or more additional episodes in their lifetime. Indicatively, approximately 80% of those with a history of two episodes will have further recurrence 3, 4. Once a first episode occurs, recurrent episodes will usually begin within five years. In general, individuals with a history of depression will have five to nine 5 separate major depressive episodes (MDEs) over their lifetime.

MDD is an illness with a well-established negative impact and is predicted to become the second-leading contributor to global burden of disease by the year 2020 6. This critical impact is mainly due to the recurrent nature of MDD, which results in a severe burden to patients, families, public health, and society 7, 8, 4.

*The current thesis uses the definitions of remission, recovery, relapse and recurrence. Recurrence, on the other hand, occurs when an individual experiences a new depressive episode after a full recovery had been achieved. The distinction between relapse and recurrence makes conceptual sense, and clear operational criteria have been proposed by Frank et al. 9.
MDD also has a significant economic impact. Not only with regard to direct cost for treatment, but also for the sizeable indirect costs in terms of sick leave and early retirement. In 2005, the combined direct cost (outpatient care, drug cost and hospitalization) and indirect costs due to morbidity and mortality of depression in Europe was estimated at €120 billion.

A promising strategy to reduce the disabling effects of depression is prevention, both primary and secondary. This thesis will focus on secondary prevention strategies in patients at high-risk of recurrence MDD. In their review, Burcusa and Iacono discuss a number of hypotheses that have been offered as explanation for the high risk of recurrences. One of these is the vulnerability-accumulation (or scarring) hypothesis, which postulates that the experience of a depressive episode induces a lasting increase in vulnerability, which increases the risk of recurrence. One aspect of the scarring hypothesis is related to the phenomena of “kindling”, which assumes that with each subsequent episode, less stress is required to provoke a new episode.

A more biological explanation of scarring suggests ‘episode sensitization’. This means that after each new episode of depression, it takes a less dysphoric mood to produce the following episode. This change is believed to be encoded at the level of gene expression. It suggests that the neurobiology of this affective disorder is a dynamic process that changes over the course of the illness.

Many variables seem to be related to risk for recurrence of MDD. However, these presumed “risk factors” could actually be manifestations of an underlying premorbid susceptibility to MDD in general. So contrary to the scarring hypothesis, Burcusa & Iacono suggest that “…individuals at high risk for multiple episodes possess the necessary characteristics to make them prone to recurrent depression, and such characteristics exist even before their first episode”. More specifically, scarring theories do not take into account that the “cut-off” for MDD risk may have already been met even before the first episode.

It could be that individuals inherit a level of risk for recurrent MDD. If they are high in this underlying genetic vulnerability, they are also likely to have an earlier age at onset, a greater numbers of episodes, increased severity of the episodes, and greater comorbidity and risk for CVD. It is not yet clear how genetic and biological factors specifically contribute to the recurrent subtype of MDD and so these factors merit further investigation as hopefully genetic risk, in future, can be modified through environmental mechanisms in order to alter the course of recurrences.
In this thesis we consider the previously presented explanatory models for the high risk for recurrence, specifically: (I) the vulnerability-accumulation (or scarring) hypothesis and (II) the premorbid vulnerability hypothesis. Our aim is to investigate premorbid factors (e.g. genetics, childhood trauma) that are present before MDD onset, as well as biological factors that could play a premorbid role and/or are involved in vulnerability-accumulation (‘scarring’). These biological variables are collected in different stadia in the course of recurrent MDD.

Genetic factors
Recurrent MDD is thought to have a higher heritability than other forms of MDD. Individuals with recurrent MDD not only have greater numbers of episodes in their lifetime but also are more likely to experience stressful life events, display increased neuroticism, have a greater risk for earlier onset of co-morbid psychopathology, and show a family history of MDD. Therefore, value might be added to a diagnosis, such as recurrent MDD, if an underlying pathophysiology or biomarker can be identified.

Risk factors for recurrence
Identifying predictors for recurrence in recurrent depressed patients is important for a better understanding of the course of this disease. These risk factors can be divided into those that can be modified and those that cannot. Identification of dynamic, potentially modifiable, (neuro) biological risk factors for recurrence could be helpful particularly in the development of targeted preventive intervention. Currently well-known risk factors for recurrence are: clinical variables (age of onset of the first episode, severity of first episode, number of previous episodes, residual symptoms), family history, negative/extreme cognitions, personality traits (neuroticism), exposure to a stress-inducing environment (daily hassles), poor social support, and maladaptive coping techniques. However, these predictors explain only part of the variation in recurrence. In the DELTA study, only the following risk factors for relapse/recurrence were significant over a 5.5 year period: a high number of previous episodes, greater residual depressive symptomatology and psychopathology, and daily exposure to a stress-inducing environment.

MDD and cardiovascular disease: a collaborative relationship.
MDD is associated with significantly elevated risk of early death. Partly because MDD has a high risk for suicide, but also because it is significantly associated with a wide variety of chronic physical disorders including arthritis, asthma, cancer, diabetes, hypertension, chronic respiratory disorders, chronic pain conditions and CVD. This thesis will focus, in part, on the association between MDD and CVD as it is of considerable relevance to both the patient and public health with regard to the costs (personal and financial) of MDD.
Different explanatory mechanisms for the proposed MDD-CVD association:

(I) As a unidirectional relationship. MDD can be considered as a risk factor for CVD, with its associated financial costs, impairments, and increased mortality risk. This is supported by meta-analyses of longitudinal studies that show that MDD is a consistent predictor of the subsequent first onset of coronary artery disease and for stroke, diabetes, and myocardial infarctions. Based on the aforementioned findings, a number of mechanisms have been proposed to explain the prospective associations of MDD with CVD. These include a variety of health behaviors known to be linked to MDD, such as elevated rates of smoking and drinking, obesity, physical inactivity, low compliance with treatment regimens. However, the question remains of whether CVD-risk is premorbid present, due to an underlying pathophysiological mechanism, or that CVD can be caused by MDD and its recurrences and thereby of an accumulation of vulnerability (scarring).

(II) As a bidirectional relationship. Some CVD-risk factors, such as obesity, increase the risk of MDD and, in turn, MDD increases the risk for the development of obesity. This relationship could explain the observed association between MDD and CVD. However, pathophysiological mechanisms underlying the mutual association between MDD and CVD are complex and still largely unknown.

(III) Through a shared risk factor. A common risk factor of MDD and CVD is thought to be metabolic syndrome (MetS*). MetS is defined as a conglomerate of metabolic risk factors associated with the development of type 2 diabetes mellitus, coronary artery disease and increased cardiovascular mortality. Major depressive disorder (MDD) has been associated with an increased incidence of type 2 diabetes mellitus and cardiovascular disorders. Thereby providing a potential shared mechanism between depression and incident somatic conditions. Pan et al. found depression and MetS to be modestly associated (unadjusted OR = 1.42; adjusted OR = 1.34) in a systematic review. Although there is continuing debate regarding standardizing the MetS symptoms and diagnostic criteria, this clustering of risk factors is unequivocally linked to an increased risk for developing CVD. To avoid obfuscation this thesis will refer to the risk factors for MetS as CVD-risk factors. Assies et al. argue that oxidative stress represents a major common biological denominator underlying psychiatric disease (e.g. MDD) and CVD. The common risk factor model implies that (part of) the observed relation between MDD and CVD is not caused by a causal relation between the two conditions.

*Five criteria: waist circumference (WC) >102 cm in men; >88 cm in women, triglyceride levels (TG) ≥150 mg/dL (1.7 mmol/L), HDL-C <40 mg/dL (1.08 mmol/L) in men and <50 mg/dL (1.3 mmol/L) in women, blood pressure (BP) ≥135/ ≥85 mmHg and fasting blood glucose (GL) ≥100 mg/dL (≥5.6 mmol/L) or known diabetes.
In sum, because both recurrent MDD and CVD-risk factors constitute significant public health challenges, studying the association between these two conditions is of high relevance and importance. Particularly as evidence of critical (neuro)biological consequences of the supposed underlying condition of recurrent MDD is emerging. This thesis was produced both at the time of- and in response to the scarcity of data available with respect to the interplay of recurrent MDD and CVD-risk factors. The goal of this thesis is to advance this burgeoning field by investigating some of the possible involved pathophysiological mechanisms that could mediate the manifestations of CVD-risk factors and stress in recurrent MDD.

**Hypothalamic-pituitary-adrenal axis**

An important biological system that might link MDD and CVD is the hypothalamic-pituitary-adrenal (HPA) axis. Dysfunction of the HPA-axis may contribute to the pathogenesis of both MDD and co-morbid CVD. This contribution may be mediated, at least partly, by the loss of glucocorticoid receptor-mediated negative feedback on inflammatory signaling. Furthermore, elevated cortisol levels in MDD could increase CVD-risk, since cortisol increases visceral fat, which is a CVD-risk factor.

Dysfunction of the HPA-axis during MDD has been called one of the most reliable finding in all of biological psychiatry. Hundreds of studies have compared HPA axis function between depressed and non-depressed individuals. However, few studies have examined the predictive value of HPA activity on course in recurrent and chronic depression (for a comprehensive meta-analysis see 41). Moreover, knowledge of the degree of (hyper/hypo)activity and the clinical conditions under which it occurs remains incomplete.

It is debate whether abnormal HPA-axis activity in MDD-patients reflects a state (i.e occurs only during MDEs), and/or represents a persistent trait. The answer to this question has pathogenic implications. If HPA-axis abnormalities are state-dependent, they might be the consequence of epiphenomenal effects of depressive symptoms or underlying/accompanying daily hassles. Conversely, if HPA-axis abnormalities are a trait, they might be genetically regulated (endophenotype) and/or mediated by perinatal programming, traumatizing childhood life-events (CLEs), and/or or scarring-effects of previous MDEs.

A major liability of the HPA-axis theory of MDD is the difficulty of defining the association of stress to MDD. As discussed, about half of the patients have a single lifetime depressive episode, whereas the other half has a recurrent course. In addition various types of acute stress, early...
childhood trauma, or long-term psychosocial problems may be involved and may lead to different responses of the stress (HPA-) system. Stress can be causative in some cases and secondary to depressed mood in others. There are also indications that stress and childhood trauma might affect the predictive value of the HPA-axis on evaluating risk of recurrence.

As discussed, the underlying pathophysiological mechanism of the recurrent nature of MDD and its association with CVD-risk needs further clarification. To help reveal whether HPA-dysfunction is a vulnerability in recurrent MDD and/or vulnerability-accumulation (scarring), we aim to investigate HPA-axis functioning in MDD-R patients.

Fatty acid metabolism

Severus et al. were the first to draw attention to the interaction between fatty acids (FA's), homocysteine, and increased mortality due to CVD-risk in MDD-patients. Current evidence suggests that a deficiency in membrane omega-3 (n-3) FA's may be a preventable risk factor for both CVD and MDD. There are several biological mechanisms that could potentially link the membrane n-3 FA deficiency observed in MDD to increased risk for CVD. In addition to clinical and preclinical evidence that n-3 FAs protect against cardiac arrhythmias, other mechanisms including enhanced platelet reactivity and aggregation, elevated triglyceride levels, and inflammation have been found.

The (cellular) membrane is a complex structure, composed primarily of a phospholipid bilayer and its constituent FAs, which provide scaffolding for proteins responsible for many key functions in the membrane. The membrane is also a natural intersection between genetic and environmental factors. Membrane defects, such as those induced by decreased docosahexaenoic acid DHA in phospholipids, can significantly alter a broad range of membrane functions. FAs are key components of (nerve) cell membrane phospholipids and synapses and are responsible for: signal transduction, ion transport and receptor sensitivity (e.g. for serotonin, dopamine, endocannabinoids). Therefore, alterations in key neurotransmitters involved in MDD can both be modified by- and contribute to oxidative stress and membrane dysfunction. This makes fatty acids (FAs) essential components of cell membranes.

Fatty acids can be classified into three families: saturated FAs, mono-unsaturated FAs and poly-unsaturated FAs (PUFAs). Unsaturated FAs have one or more double bonds between carbon atoms. The position of the double bond in the carbon chain is included in the name of the unsaturated FA. When the double bond is in position 6, the unsaturated FA is called an
‘omega-6 FAs’ and those with a double bond in position 3 are called ‘omega-3 FAs’, examples of omega-3 FA’s include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In a typical western diet, omega-6 FAs are much more abundant than omega-3 FAs. A high omega-6 to omega-3 ratio can alter cell membrane properties and increase production of inflammatory mediators because arachidonic acid, an omega-6 FA found in cell membranes, is the precursor of inflammatory eicosanoids, such as prostaglandins and thromboxanes. By contrast, omega-3 FAs are anti-inflammatory. Therefore, a high dietary omega-6 to omega-3 fatty ratio could promote neuroinflammation. Decreased omega-3 FAs concentration in the diet may also act by altering central nervous system cell membrane fluidity and phospholipid composition, which may alter the structure and function of the proteins embedded in e.g. neurotransmitter receptors 53, which can be the case in MDD. Furthermore, FAs alterations 54 have been implicated in both CVD and MDD. It is of note that this ratio has increased dramatically throughout history.

A genetic factor could underlie FA-alterations in MDD-R. This is suggested by the bimodal distribution of factors reflecting FA-metabolism in MDD-R patients 55 implying a dichotomous causal factor (e.g. a genetic mutation leading to altered enzymatic processing of dietary FA’s). An interesting candidate for the factor is the fatty acid-binding protein 2 (FABP2) gene. It is mostly expressed in small intestine enterocytes, where it codes for intestinal FABP, which is accountable for uptake of dietary FAs. A transition G to A at FABP2-codon 54 results in an amino acid substitution (Ala54 to Thr54) 56. This polymorphism is common, with a Thr54 allelic frequency of 30% in most populations, resulting in altered FABP FA-affinity. Homozygous Thr54-carriers show altered dietary FA-uptake, with increased postprandial concentrations of 14-18-carbon FAs 57. As a result of the (patho)physiological role of FAs in metabolism, they have been suggested as an explanatory factor for the association of FABP2 with increased insulin resistance and FA-oxidation, which corroborates observations suggesting a role of the FABP2 Ala54Thr-polymorphism in CVD-etiology (e.g. obesity and atherosclerosis) 58, 59.

With respect to MDD, an important limitation of studies so far on FA levels is that they mostly addressed only n-3 and n-6 PUFA levels. They did not measure the whole FA spectrum. Neither were estimates of their respective desaturases and elongases routinely reported 49, 60, 61. Moreover, these studies focused mainly on patients suffering from a single depressive episode while data regarding the FA metabolism of patients with recurrent depression is currently lacking.
In this thesis we compare PUFA-levels in patients with MDD-R - to PUFA-levels in non-depressed controls and we investigate whether PUFA-levels are associated to the current depressive status. Moreover, we investigate whether the Thr54-polymorphism in the \textit{FABP2}-gene is (I) more prevalent in MDD-R patients than in sex- and age-matched controls, (II) associated with observed alterations in FA-metabolism, and (III) associated with crucial CVD-risk factors, such as waist circumference.

\textbf{One Carbon metabolism}

Another biological pathway potentially underlying susceptibility to onset, symptomatology and recurrence of MDD is the folate-mediated one-carbon (1-C) metabolism \textsuperscript{62, 63}. An inconsistent pattern emerges when looking at the associations between MDD and the key constituents of the 1-C-cycle (homocysteine folate, vitamin B\textsubscript{6} and vitamin B\textsubscript{12}) \textsuperscript{62, 64, 65}. The 1-carbon cycle is also associated with increased CVD-risk in patients with MDD \textsuperscript{66}. A meta-analysis concluded that each 5 micromol/L increase in homocysteine independently raised CVD-risk by approximately 20\% \textsuperscript{67}. In addition, a meta-analysis of prospective studies showed that folate level is inversely associated with CVD-risk \textsuperscript{68}.

The 1-C-cycle plays a central role in (I) the regulation of oxidative stress and (II) the generation of methyl groups for methylation of DNA, proteins, phospholipids and neurotransmitters \textsuperscript{69, 70}. A crucial enzyme in this pathway is 5,10-methylenetetrahydrofolate reductase (\textit{MTHFR}). A single nucleotide polymorphism (SNP) in the \textit{MTHFR} gene (C677T or rs1801133) results in the production of a thermolabile variant of \textit{MTHFR}, which is associated with decreased methylation capacity \textsuperscript{71} and increased oxidative stress. This genetically determined variation in 1-C-cycle activity is associated with increased stress sensitivity, due to a multitude of factors (e.g. a lower level of the important intracellular antioxidant glutathione). This genetic variation may therefore contribute to alterations in neurocognitive functioning and mood regulation, predisposing to either development and/or the adverse course of MDD \textsuperscript{72, 73}.

Evidence for the link between polymorphisms in the \textit{MTHFR} gene with MDD is thus far equivocal. Patients with the \textit{MTHFR} 677T genotype may be particularly prone to recurrent MDD as a result of a dysfunctional methylation metabolic pathway and/or long-lasting (methylation) effects of childhood traumatic stress. The association of the \textit{MTHFR} gene with recurrent MDD, however, has not yet been investigated. Studying genetic susceptibility to stress is of specific relevance in the context of MDD as stress is considered one of the main pathogenic factors involved in MDD recurrence.
Most genetic *MTHFR* studies in patients with MDD did not simultaneously measure the key constituents of the 1-C-cycle mentioned above (folate, vitamin B<sub>6</sub>, B<sub>12</sub> and homocysteine). The *MTHFR* 677TT genotype and, to a lesser extent the 677CT genotype, is associated with a pattern of significant elevation in the circulating concentrations of the thiol amino acid homocysteine and a decrease in serum folate concentrations, which may parallel a similar reduction in 5-*MTHF* in the central nervous system (CNS).

Total homocysteine can be interpreted as a marker of oxidative stress resulting in neurological and vascular damage and as an interruption of the optimal biosynthesis of neurotransmitters. High homocysteine levels have been linked with MDD. Low folate concentrations have been found in patients with MDD compared to healthy controls. There is also evidence that vitamin B<sub>12</sub> and vitamin B<sub>6</sub> may be associated with depressive symptomatology. Nevertheless, not all studies have found significant associations between the 1-C-cycle constituents and depressive symptomatology.

In this thesis, we will examine integratively all the 1-C components, while including the possible effects of medication and depressive state, and thereby attempt to provide more insight in the possible association between the *MTHFR* polymorphism and recurrent MDD. Additionally, we investigated if C677T *MTHFR* could be a potential predictor for depressive symptomatology and MDD recurrence in the context of traumatic stress during early life.

Medically Unexplained Physical Symptoms

Somatic symptoms, unattributed to a diagnosable medical condition, are common in patients with MDD. These medically unexplained physical symptoms (MUPS) show a wide variety of severity ranging from single, mild, and transient to a larger number of more chronic and extremely disabling symptoms. High levels of MUPS in MDD patients may greatly impair their quality of life and could increase the burden of depression, hinder full remission, and may hamper treatment response. Understanding the relation between MUPS and MDD is therefore of high relevance. A correlation between depression and MUPS has been reported in cross sectional studies but longitudinal studies investigating the temporal relationship between depression and MUPS are scarce.

Mutual underlying biological pathways could (partly) play a role in the relation between recurrent MDD and MUPS. The first candidate is fatty acid (FA)-metabolism as FA’s play multiple roles that
include: (I) participating in immune regulation (II) determining neuronal membrane stability, and (III) being involved in neurotransmission and signal transduction. MDD is linked to lowered n-3 FAs levels and an imbalance between n-3/n-6 levels, which is viewed as harmful and might be linked to somatic manifestations. A second candidate is the serotonergic pathway because it is considered to play a role in both MDD and the development of pain symptoms. There is some evidence for an association of longer 5-HTTLPR allele mutations with MUPS, while MDD itself is related either with a longer 5-HTTLPR allele mutation or other 5-HTTLPR mutational variants. Additionally, lowered n-3 FA status is related to serotonergic disturbances and could be a pathway for mood and cognitive dysfunction in depression.

In this thesis, we assess whether MUPS is a predictor of recurrence in patients with recurrent depression. This could be of clinically relevance, especially because MUPS may represent a dynamic modifiable factor involved in recurrence. Additionally, the associations with FA- and serotonergic pathways and MUPS will be explored.

1.1.2 Study used in this thesis (DELTA study)
This thesis is based on data from two research projects that were initiated and conducted by the Program for Mood Disorders at the Academic Medical Center (AMC) located in Amsterdam, Netherlands.

First, data was drawn from the DELTA (Depression Evaluation Longitudinal Therapy Assessment) Study, which was initiated with a grant from the Health Research Development Council, Department Prevention Program. The aim of this project was to assess the recurrence-preventing effect of cognitive therapy (CT) in recurrent MDD in a randomized controlled trial (ISRCTN 68246470 http://www.controlled-trials.com/ISRCTN68246470/bockting).

The second source of data for this thesis was drawn from an additional biological study within the DELTA study entitled “Somatic, psychological and social course in patients with recurrent MDD: development of clinical relevant models”, which received support from the Netherlands Foundation for Mental Health.

Studying recurrent-MDD is important because it represents a more biologically determined MDD-subtype, which may be specifically linked to recurrence and CVD-risk. For this reason, we expected these patients to deviate strongly from healthy controls in the (patho)physiological and genetic mechanisms and to have more pronounced alterations in their course of
MDD. Moreover, this patient group can be considered characteristic for those patients particularly causing the large MDD-associated burden of disease. Interestingly, few in past studies have included this patient group.

A full description of DELTA has been provided by Bockting \textsuperscript{86}, \textit{The rhythm of depression}.

In addition to these two research projects, we recruited an age and gender matched, healthy, non-depressed control group by advertising.
1.1.3 Aims and outline of this thesis (including figure)
The general aim of this thesis is to investigate aspects of biological- and pathophysiological mechanisms (including HPA-axis functioning, one-carbon and fatty-acid metabolism), the role of stress (childhood trauma, life events and daily hassles), and the impact of gene-environment (stress, childhood trauma) interactions associated with the course of recurrent MDD. This thesis aims to assess this in relation to: (I) clinical relevance, (II) underlying associated mechanisms and course of depression in terms of relapse and recurrence and (III) improvement of treatment strategies to prevent recurrences.

These aims resulted in the following research questions:
1  What is the relation between recurrent depression, being overweight, and obesity in patients with MDD-R and is this relation explained or modified by use of anti-depressant medication (AD)?

Chapter 2 describes the results of a study that focused on the relation between obesity and MDD-R and the association between long-term use of ADs and obesity. Additional data were derived from a large Dutch population-based study of time trends on obesity prevalence rates.

2a In what ways does the HPA-axis functioning differ between MDD-R patients and healthy controls?
2b Does this reflect a persistent trait or is this influenced by depressive state, stress or previous episodes associated with recurrence? c) Can this relation be modulated by cognitive therapy?

Chapter 3.1 describes the results of our longitudinal study that assessed the HPA-axis activity over time in highly recurrent MDD-patients. At the onset of the study, all patients were in remission and were matched with those in the healthy control group. The patients were followed-up prospectively at three months and two years, where MDD-recurrence and HPA-axis activity data was collected. This longitudinal design was chosen to distinguish between state and trait effects.

3 Do HPA-axis measures predict time to recurrence in MDD-R patients?
In chapter 3.2, we investigate whether HPA-axis measures predict time to recurrence in remitted recurrently depressed patients corrected for residual depressive symptoms. We also examine the role of stress (current daily hassles) and childhood trauma on the predictive value of the HPA-axis on recurrence.
4a Do polyunsaturated fatty acid (PUFA) levels and the n-6/ n-3 ratio differ between MDD-R patients compared to non-depressed controls?

4b Are these possible alterations state dependent, or do they reflect a trait (i.e. are they independent of the current depressive status)?

Chapter 4.1 describes our explorative study that comprised of 44 randomly selected subjects chosen out of a cohort of 134 patients with the recurrent form of MDD (MDD-R). In this study, we assessed homocysteine levels together with saturated fatty acids (FAs), monounsaturated fatty acids (MUFAs) and polyunsaturated FAs (PUFAs) of the omega n-3, omega-6 and omega-9 series in plasma and erythrocytes. Levels were compared with laboratory reference values.

We subsequently carried out a case-control study, which is described in chapter 4.2. The sample for this study consisted of 137 patients with MDD-R and 65 matched non-depressed controls.

5a What is the relationship of the Ala54Thr fatty acid-binding protein 2 (FABP2) polymorphism in recurrent depression?

5b Are fatty acid concentrations associated with the CVD-risk factor waist circumference?

In chapter 4.3, we investigate whether the Thr54-polymorphism in the FABP2-gene is (I) more prevalent in MDD-R patients than in sex-and age-matched controls, (II) associated with observed alterations in FA-metabolism, and (III) associated with the CVD-risk factor, i.e. waist circumference.

6 What is the prevalence of the MTHFR polymorphism and its relationship with 1-C-cycle components in MDD-R patients compared to matched controls and are the latter influenced by depressive state and/or AD use?

In chapter 5.1, we examine the MTHFR C677T polymorphism together with 1-C-cycle components (folate, homocysteine, vitamin B₆ and B₁₂) in clinically diagnosed patients with a high risk of recurrence of depression compared to age- and sex-matched healthy controls.

7a What is the association between recurrent MDD patients carrying the T allele with time to recurrence?

7b Is this association associated with having been exposed to traumatic childhood events (TCE)?

In chapter 5.2, we investigate the moderating effect of the C677T MTHFR variant on the association between traumatic childhood events (TCEs) and MDD recurrence in a 5.5-year follow-up study in a sample with recurrent MDD and, in an independent replication sample, on depressive symptomatology in healthy individuals from the general population.
8a What is the association between medically unexplained physical symptoms (MUPS) with time to recurrence in recurrent depression?

8b What is the association between MUPS with FA metabolism and the serotonin transporter gene?

In chapter 6, we assess the predictive value of medically unexplained physical symptoms (MUPS) on time to recurrence in recurrent depression. In addition, we examine the association between a sustained high level of MUPS and omega n-3 and n-6 fatty acid (FA)-status as well as functional polymorphisms in the promoter region of the serotonin transporter gene (5-HTTLPR), to elucidate pathophysiological mechanisms that could explain the relations between MUPS and MDD.

To conclude, in chapter 7, the findings of this thesis are summarized and discussed and implications and suggestions for future directions are presented.
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Chapter 2 Obesity
Obesity

2.1 The ‘weight’ of recurrent depression: a comparison between recurrent depressed individuals and the Dutch population.

Lok A, Visscher TLS, Koeter MWJ, Assies J, Bockting CLH, Verschuren WMM, Gill A, Schene AH

Psychother Psychosom, 2010, 79:386-388
Both depression and obesity share similar risk factors and are mutually associated. Among obese people, depression is one of the most common psychiatric disorders. Studies in persons seeking treatment for mood disorders like depression indicate that obesity and overweight are common problems in these groups. It has also been suggested that obesity and depression might be different manifestations of the same disease.

Although most publications regarding the association between major depressive disorder (MDD) and obesity corroborate each other, the literature on the relation between obesity and the recurrent type of MDD (MDD-R; having had at least 2 major depressive episodes) is limited and equivocal. Most studies on depression and obesity did not distinguish between single and recurrent episodes. However, this distinction may be important because depression is increasingly considered a chronic recurrent disorder with various levels of interepisodic functioning, and evidence is growing that the recurrent type (MDD-R) is a distinct one.

Most studies on the relation between depression and obesity did not control for antidepressant (AD) medication use, although a substantial part (20-60%) of the recurrently depressed patients use ADs for lengthy periods of time. Recently, Patten et al. reported in this journal an association between AD use and obesity incidence in their longitudinal analysis on a large community sample followed over a 10-year period. However, Patten et al. did not distinguish between single and recurrent episodes either. Our study, albeit a cross-sectional one, elaborates on their findings by focusing on the relation between obesity and MDD-R and the association between long-term use of ADs and obesity.

MDD-R patients participated in the DELTA study. To be eligible for this study, they had to meet the following criteria: (a) at least 2 major depressive episodes in the past 5 years (DSM-IV), (b) current remission status, according to DSM-IV criteria, for longer than 10 weeks and no longer than 2 years before, and (c) Hamilton Rating Scale for Depression of <10. At 2 years, follow-up assessment anthropomorphic parameters were collected of 134 subjects. The protocol was approved by the ethics review committees. Reference data of BMI, waist-to-hip ratio and waist circumference were derived from the cross-sectional monitoring project on risk factors for chronic diseases (MORGEN project), a large population-based study of time trends on obesity prevalence rates. To adjust for differences in age and gender distribution between the reference and patient groups, we used for each sex the proportions of people constituting a specific age cohort in the total Dutch population as weights in the calculation of both the reference group and patient group anthropometric parameters for each sex.
a method resembling direct standardization. All subjects had their weight (in kilograms), height, waist and hip (in centimetres) measured by trained staff.

To assess relapse/recurrence, the Structured Clinical Interview for DSM-I V (SCID-I) 13 was used. In the DELTA study, use of ADs was recorded but not controlled by the investigators for obvious ethical reasons. Every 3 months during the 2-year study, information on AD (type and dosage) over the previous month had been monitored using the Trimbos/IMTA Self-Report Questionnaire for Cost Associated with Psychiatric Illness 14 which covers a maximum recall period of 1 month. Regarding the use of ADs, two groups were distinguished 7: those who used ADs throughout the entire 2-year study period (n = 46) and those who did not use ADs continuously, but intermittently (n = 49) or not at all (n = 39). Differences between these groups in BMI, waist circumference and waist-to-hip ratio were tested stratified by gender.

Standardized prevalence rates of (abdominal) overweight and obesity in recurrent depression patients and the reference group are shown in table 1. Overweight and obesity occurred more often in patients with recurrent depression than in the reference group, although statistical significance was reached in women only (74% of this sample). The extent of the differences was also larger for women (medium effect sizes in terms of Cohen’s $h$ 15).

Table 1. Prevalence rates of (abdominal) overweight and obesity in recurrent depression patients and the general population

<table>
<thead>
<tr>
<th></th>
<th>Recurrent depression</th>
<th>General population</th>
<th>Difference</th>
<th>$p$</th>
<th>95% CI</th>
<th>Cohen’s $h$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Men</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overweight (BMI ≥25 kg/m²)</td>
<td>62.2</td>
<td>46.5</td>
<td>15.7</td>
<td>0.152</td>
<td>-5.8-37.1</td>
<td>0.32</td>
</tr>
<tr>
<td>Obesity (BMI ≥30 kg/m²)</td>
<td>16.4</td>
<td>8.5</td>
<td>7.9</td>
<td>0.408</td>
<td>-10.8-26.5</td>
<td>0.24</td>
</tr>
<tr>
<td>Abdominal overweight (WC ≥94 cm)</td>
<td>53.9</td>
<td>35.9</td>
<td>17.9</td>
<td>0.083</td>
<td>-23.7-38.3</td>
<td>0.36</td>
</tr>
<tr>
<td>Abdominal obesity (WC ≥102 cm)</td>
<td>24.4</td>
<td>15.0</td>
<td>9.5</td>
<td>0.359</td>
<td>-10.7-29.6</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Women</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overweight (BMI ≥25 kg/m²)</td>
<td>55.6</td>
<td>35.7</td>
<td>19.9</td>
<td>0.002</td>
<td>7.6-32.4</td>
<td>0.40</td>
</tr>
<tr>
<td>Obesity (BMI ≥30 kg/m²)</td>
<td>28.0</td>
<td>9.7</td>
<td>18.3</td>
<td>0.002</td>
<td>7.1-29.5</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table is continued on the next page >
Within the MDD-R patient group, serotonin-selective reuptake inhibitors (SSRIs) were the most commonly used type of AD (73.3% of the men and 84.6% of the women) among the continuous AD users. Compared with SSRIs, other types of ADs used (e.g. tricyclic ADs) did not have a significant impact on the anthropometric measures. AD use was related to anthropometric measures. The mean AD equivalent correlated positively with both waist circumference ($r = 0.239, p = 0.006$) and waist-to-hip ratio ($r = 0.252, p = 0.004$), but not with BMI. In addition, mean waist circumference and waist-to-hip ratio scores were consistently higher amongst the continuous AD users compared to intermittent and no AD users. The mean waist circumference for non-users was 85.5 cm versus intermittent users with 87.7 cm versus continuous users with 94.5 cm ($F_{2, 122} = 4.95, p = 0.009$). The mean waist-to-hip ratio for non-users was 0.82 versus intermittent users with 0.84 versus continuous users with 0.87 ($F_{2, 122} = 6.08, p = 0.003$).

These results in our MDD-R sample are comparable to those of other studies in first episode or combined first episode MDD-R depressive patient samples. To our knowledge, this is the first study that examines this relation in recurrently depressed patients. Importantly, our patients were not in a depressive relapse per se (did not meet full DSM-IV-R criteria for MDD) at the time of assessment. Comparisons were adjusted for age and sex differences; however, we cannot rule out that the patient and reference groups are different in other characteristics that may account for the differences in anthropomorphic characteristics. However, this is also a drawback of all other studies.

Patients using ADs continuously, mostly SSRIs, show significantly more (abdominal) overweight and obesity than those using them intermittently or not at all. Compared with SSRIs, other types of ADs used (e.g. tricyclic ADs) did not have a significant impact on the anthropometric measures. We did find, however, a small association between AD equivalent dosage and waist circumference and waist-to-hip ratio. Patten et al. concluded that a major depressive

<table>
<thead>
<tr>
<th>Abdominal overweight (WC ≥80 cm)</th>
<th>70.3</th>
<th>44.1</th>
<th>26.2</th>
<th>&lt;0.001</th>
<th>14.5-37.9</th>
<th>0.54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal obesity (WC ≥88 cm)</td>
<td>40.6</td>
<td>21.3</td>
<td>19.3</td>
<td>0.002</td>
<td>7.1-31.6</td>
<td>0.42</td>
</tr>
</tbody>
</table>

**Abbreviations** - WC = Waist circumference; CI = confidence interval; Cohen’s $h = 2\arcsin(\sqrt{p1}) - 2\arcsin(\sqrt{p2})$; small $h = 0.20$, medium $h = 0.50$, large $h = 0.80$.

* Direct standardization to the age distribution in the Netherlands.

episode does not appear to increase the risk of obesity, although the results were limited by the self-report of height and weight, but they did find an association between AD use and obesity incidence. This association was, like in our study, found for SSRIs and venlafaxine. One explanation for these findings is that physicians may specifically select these medications for use in patients they believe to be most at risk of weight gain. As such, those exposed to SSRIs may represent a group at higher risk of obesity for reasons other than their AD medications. If, on the other hand, ADs act as risk factors for obesity, this is of great concern given the increase in the number of patients who receive AD treatment.

In general, a better understanding of the relationship between obesity and depression, i.e. understanding the beneficial and adverse effect of psychotropics on appetite, eating behaviour, body weight and metabolism \(^{18}\), should improve our ability to prevent and treat both obesity and depression. Thereby, ideally person-tailored interventions can be developed, including effective non-pharmaceutical preventive strategies for recurrent depression \(^{9,19,20,21}\) and extra physical activities with - as added benefit - protection against AD-induced weight gain.

Acknowledgment
This study has been made possible due to financial aid of the Netherlands Foundation for Mental Health, Department Prevention Programme (ZONMw).
Time after Time; **biological factors in the course of recurrent depression**

**References**


Chapter 3
HPA-axis
HPA-axis

3.1 Longitudinal Hypothalamic-Pituitary-Adrenal Axis trait and state effects in recurrent depression

Lok A*, Mocking RJT*, Ruhe HG, Visser I, Koeter MWJ, Assies J, Bockting CLH, Olff M,
Schene AH

* Authors contributed equally

Psychoneuroendocrinology 2012, 37(7):892-902
Summary

**Background:** Hypothalamic-pituitary-adrenal (HPA)-axis hyperactivity has been observed in (recurrent) major depressive disorder (MDD), although inconsistently and mainly cross-sectional. Longitudinal studies clarifying state-trait issues are lacking. We aimed to determine whether HPA-axis (hyper)activity in recurrent MDD is: (I) reflecting a persistent trait; (II) influenced by depressive state; (III) associated with stress or previous episodes; (IV) associated with recurrence; and (V) influenced by cognitive therapy.

**Methods:** We included 187 remitted highly recurrent MDD-patients (mean number of previous episodes: 6.3), participating in a randomized-controlled-trial investigating the preventive effect of additional cognitive therapy on recurrence. In an add-on two-staged patient-control and prospective-cohort design, we first cross-sectionally compared patients’ salivary morning and evening cortisol concentrations with 72 age- and sex-matched controls, and subsequently longitudinally followed-up the patients with repeated measures after three months and two years.

**Results:** Patients had higher cortisol concentrations than controls (p < .001), which did not change by MDD-episodes during follow-up. HPA-axis activity had no relation with daily hassles or childhood life events. Cortisol concentrations were lower in patients with more previous episodes (p = .047), but not associated with recurrence(s) during follow-up. Finally, randomly assigned cognitive therapy at study-entry enhanced cortisol declines over the day throughout the two-year follow-up (p = .052).

**Conclusions:** Our results indicate that remitted recurrent MDD-patients have a persistent trait of increased cortisol concentrations, irrespective of stress. In combination with our finding that patients’ cortisol concentrations do not change during new MDD-episodes (and thus not represent epiphenomenal or state-effects), our results support that hypercortisolemia fulfills the state-independence criterion for an endophenotype for recurrent depression.
1. Introduction

Hypothalamic-pituitary-adrenal (HPA)-axis research in major depressive disorder (MDD) has been predominantly cross-sectional in nature, comparing patients in a depressed state with healthy controls. Observations of hypersecretion of corticotrophin releasing hormone and cortisol, reduced feedback from glucocorticoids, and enlarged endocrine glands, resulted in a general consensus of an HPA-axis overdrive in patients with more severe forms of MDD. However there were also conflicting data; (I) some studies observed no elevated or even lowered cortisol concentrations in MDD-patients compared with controls; (II) glucocorticoid treatment and diseases with higher (e.g., Cushing’s), but also lower cortisol concentrations (e.g., Addison’s; posttraumatic stress disorder) are both associated with MDD; and (III) treatments with glucocorticoid receptor agonists as well as antagonists have both been proposed anti-depressogenic.

In addition, it has been increasingly recognized that MDD is a chronic recurrent disorder. Indicatively, at least 80% of clinically recovered MDD-patients will experience a recurrence during 25-years follow-up. With, on average, five subsequent major depressive episodes (MDEs), the recurrent nature of MDD is a severe burden to patients, families and societies. The predominant cross-sectional studies could not address the association of HPA-axis disturbances with this recurrent course of MDD. More recently, longitudinal studies investigated HPA-axis activity preceding, and subsequent to, the depressed state. For example, HPA-axis hyperactivity was also observed in remitted MDD-patients, although others found no differences or even hypoactivity. In addition, during transition from an acute depressive state to remission, sustained HPA-axis hyperactivity predicted recurrence during follow-up. Likewise, higher cortisol concentrations in adolescents prospectively determined MDD onset during follow-up.

Taken together these findings raise the question whether abnormal HPA-axis activity in MDD-patients reflects a state only during MDEs, and/or represents a persistent trait. This question is not merely of academic importance. For example, if HPA-axis abnormalities show to be state-dependent, these abnormalities could mediate some of the MDE-symptoms and scarring effects. Proven true, treatment during a MDE directed at normalizing HPA-axis activity could reduce these symptoms and prevent scarring. On the other hand, if HPA-axis abnormalities show to be a trait, they could be involved in the pathogenesis of a new or recurrent MDE. If so, preventive treatment directed at normalizing HPA-axis activity could be indicated. For example, cognitive therapy could be a promising candidate as it was shown to protect against recurrences and to normalize HPA-axis activity.
Besides these clinical aspects of the state-trait discussion, it also poses pathogenetic issues. If HPA-axis abnormalities show to be state-dependent, they might be the consequence of epiphenomenal effects of depressive symptoms or accompanying daily hassles. On the contrary, if HPA-axis abnormalities show to be a trait, they might be the consequence of traumatizing childhood life-events (CLEs), scarring-effects of previous MDEs and/or perinatal programming, but could also be genetically regulated (endophenotype). This latter hypothesis is strengthened by previous research showing evidence that fulfilled the following endophenotype criteria: familial association, cosegregation, and heritability. However, the endophenotype state-independence criterion, i.e., “manifests in an individual whether or not illness is active”, has, to our knowledge, not yet been addressed for HPA-axis activity in MDD.

To further clarify these state-trait issues, we performed a longitudinal study to assess HPA-axis activity over time in highly recurrent MDD-patients. At study entry all patients were in remission, and compared with a matched control group. Subsequently, the patients were followed-up prospectively at three months and two years, while MDD-recurrence and HPA-axis activity were monitored.

We hypothesized that in patients with recurrent MDD HPA-axis hyperactivity: (I) reflects a trait, i.e. remitted patients exhibit higher cortisol concentrations compared with controls; (II) is additionally influenced by depressive state (i.e. more outspoken HPA-axis abnormalities during a recurrent MDE at follow-up); (III) is associated with (a) current daily hassles, (b) CLEs, and (c) number of previous MDEs. Furthermore, we hypothesized that: (IV) cortisol concentrations are higher in patients who experience recurrence(s), compared with patients who remain in remission during the entire follow-up period; and finally that (V) preventive cognitive therapy normalizes heightened HPA-axis activity.

2. Methods and materials
2.1. Design
The patient sample used in this study was recruited at psychiatric centers and through media announcements to participate in a randomized controlled trial assessing recurrence-preventing effects of cognitive therapy (CT) in recurrent MDD. We used an add-on twostaged case-control and prospective-cohort design. First, we cross-sectionally compared patients with controls at study entry (T0) in the case-control stage. Subsequently we longitudinally followed-up the patients with repeated measures at three months (T1; after the CT-intervention period) and
two years (T2) in the prospective-cohort stage, to assess short- and long-term (I) stability of patients’ characteristics and (II) effects of CT.

We allocated eligible patients to treatment as usual or to an additional preventive CT-module. This module consisted of eight weekly group sessions, focusing on dysfunctional attitude identification and change. Treatment as usual involved ‘naturalistic’ care, ranging from continuous antidepressant use to no treatment at all. The study was approved by the ethics committee of the Academic Medical Center of the University of Amsterdam. All subjects provided written informed consent.

2.2. Study sample

2.2.1. Patients
We included remitted MDD-patients (18-65 years), who had experienced ≥2 MDEs in the last five years, according to the DSM-IV and assessed by trained evaluators using the Structured Clinical Interview for DSM-IV disorders (SCID). Patients had to have reached remission status >10 weeks and <2 years ago. We defined remission according to DSM-IV criteria and a score ≤9 on the 17-item Hamilton Depression Rating Scale (HDRS17). We excluded subjects with: (a history of) bipolar spectrum disorder; (a history of) any psychotic disorder; organic brain damage; alcohol and/or drug abuse and/or dependency; or predominant anxiety disorder, all assessed using the SCID. Furthermore, current steroid use was also an exclusion criterion.

2.2.2. Controls
We recruited age- and sex-matched controls by advertisements in a diversity of newspapers and magazines. Controls had to have no current or past (personal and/or family) history of psychiatric axis-I disorders according to the DSMIV (assessed with the SCID). Furthermore, current steroid use was an exclusion criterion for the controls as well.

2.3. Study measurements

2.3.1. Depression characteristics and covariates
For the case-control stage of our study, at T0, we determined educational level (low, middle, and high), anthropometric measures (body mass index, waist and hip circumference), smoking behavior and medication use (including contraceptives) for both patients and controls.

During the prospective-cohort stage involving the patients, we assessed the number of previous MDEs at T0. As described previously, the range of previous MDEs was 2-70
(median = 4, interquartile range = 3), and not normally distributed, which could not be resolved by transformation. To test the effects of previous MDEs on HPA-axis activity, we therefore dichotomized the variable previous MDEs. We chose a cut-off point that created the most equally numbered groups of patients, to maximize conceivable power and/or contrast. The optimal cut-off point was the median (4), with 59.4% of the patients having <5 previous MDEs and 40.6% having ≥5 MDEs. In addition, we assessed CLEs before the age of 16 with the 15-item Negative Life Events Questionnaire (which we dichotomized; experienced CLEs yes/no) 32. Events may involve the participant or significant others. This questionnaire proved to have a good predictive validity, as the number of negative life events predicted MDD-symptom severity 33. We measured daily hassles at T0, with the 114-item Everyday Problem Checklist, providing a continuous score 34. Finally we assessed MDD-symptoms, in addition to the SCID, with the Beck Depression Inventory (BDI), at T0 35.

During follow-up, we repeated the assessments of daily hassles and MDD-symptoms at T1 and T2, with the Everyday Problem Checklist, BDI and SCID. With these follow-up assessments of the SCID we diagnosed relapses (<6 months after a previous MDE) or recurrences during follow-up, both further addressed as ‘recurrence’ for clarity reasons. Furthermore, during the whole follow-up, from T0 to T2, we monitored antidepressant medication by using the Trim-bos/IMTA Self Report Questionnaire for Costs Associated With Psychiatric Illness, every three months 36. To make data manageable for analyses, we operationalized antidepressant use as continuous antidepressant use during follow-up (yes/no). The non-continuous group both included patients who took antidepressants intermittently and patients that did not take antidepressants at all 37.

2.3.2. Hormone measures
For the case-control stage of the study, patients and controls collected saliva with neutral cotton salivettes (Sarstedt AG and Co, Nümbrecht, Germany) at home at three sampling moments on two consecutive days (day one: 0800 h and 2200 h; day two: 0800 h). For the prospective-cohort stage, we repeated the T0 measures with follow-up measures at T1 and T2 in patients only. Saliva reliably reflects blood cortisol concentrations, in a relatively stress-free and minimally intrusive way 38. We instructed subjects to rinse their mouth with water and not to brush their teeth before sampling. Subjects collected morning samples after an overnight fast, and kept the samples in the refrigerator until they sent them back by mail on day two. We stored samples at -20 °C until analysis by radioimmunoassay (IBL Hamburg; designed for saliva samples). Intra- and interassay variations were 5.1% and 6.5%, respectively.
2.4. Statistical analysis

2.4.1. Data cleaning, imputation

We assigned cortisol concentrations that exceeded four standard deviations from the mean as missing, because this suggests blood contamination. To reduce bias potentially introduced by missing values, we used a multiple imputation technique using the package Amelia II. Multiple imputation is considered the state-of-the-art way to handle missing values, and results in correctly estimated standard errors and confidence intervals. We used multiple imputation separately for the cross-sectional comparison between patients and controls and for the longitudinal analysis that only applied to the patients. Imputation resulted in five imputed datasets for the cross-sectional case-control analyses (imputation one) and five imputed data sets for the longitudinal analyses of the patient-cohort (imputation two). After imputation for T0, T1 and T2, we calculated the mean of the two morning cortisol concentrations (day one and two), since variability between morning measures on the two consecutive days was equal for patients and controls. All cortisol values showed normal distributions after log transformations, which we used in all analyses.

2.4.2. Subject characteristics and propensity scores

We compared patients’ and controls’ baseline characteristics using χ² and Student’s t-test statistics. In further analyses we adjusted for confounders using propensity scores, representing the predicted probability for a case to belong to a certain group (e.g. patient or control), calculated in a binary logistic model with the chosen confounders as predictors. This way, we could correct for multiple confounders in one score at the same time without substantial loss of power. We calculated a propensity score for comparisons between patients and controls that corrects for common confounders: sex, age, educational level, contraceptive use, steroid use in the month before assessment, smoking, weight and waist and hip circumference (PS₁). We created PS₂ to adjust the effect estimates in the longitudinal analyses, which, in addition to the confounders in PS₁, also corrects for the potential confounders: follow-up alcohol and drug use (yes/no), benzodiazepine therapy (yes/no), receiving CT treatment (yes/no) and continuous antidepressant use (yes/no).

2.4.3. Models to distinguish trait and state-effects

To assess whether HPA-axis disturbances are a trait in MDD, we used linear mixed models, with cortisol as the dependent variable and sampling moment (morning/evening), group (patient/control) and the moment × group-interaction as independent variables. We used linear mixed models to incorporate correlations between repeated measurements in the same subject,
thereby boosting power, and to achieve flexibility to model time effects. In case the moment × group-interaction was non-significant, we removed this term and used the remaining more parsimonious model. We adjusted for confounders by adding PS, to the final model.

To assess the effect of depressive state on cortisol, we modeled depressive state as a time-dependent covariate. We tested a linear mixed model using the longitudinal repeated measures patients’ data with cortisol (T0, T1 and T2) as the dependent variable, and follow-up time (T0, T1 and T2), sampling moment (morning/evening), follow-up × moment-interaction, depressive state (indicated by the SCID at T0, T1 and T2) and the state × moment-interaction as independent variables.

2.4.4. Additional analyses
To assess the association of CLEs (yes/no), number of previous MDEs (≥5 previous MDEs yes/no), and the occurrence of recurrence during follow-up (yes/no), with HPA-axis activity, we one by one included these factors in subsequent models with cortisol as dependent variable, and follow-up time (T0, T1 and T2), sampling moment (morning/evening), follow-up × moment-interaction, “factor” (yes/no), “factor” × moment-interaction, and “factor” × follow-up-interaction as independent variables. When a higher order interaction did not contributed significantly to the model, we removed this term and used the resulting more parsimonious model. We determined the effect of daily hassles on HPA-axis activity with a comparable model as the one that was used for the state-effect, where the continuous Everyday Problem Checklist score replaced the statefactor as a time-dependent variable. We adjusted for confounders by incorporating PS to the model.

To determine the effects of CT on HPA-axis activity, we first tested whether the two randomized groups (CT yes/no) were comparable on T0 (before CT). We then assessed the CT effect on cortisol at T1 and T2 (after CT).

We used PASW statistics 18.0 (SPSS, Inc., 2009, Chicago, IL). For multivariate estimates we combined separate significance tests for the five imputed datasets into one pooled test with a SPSS macro from van Ginkel (2006). We considered \( p < .05 \) statistically significant.
3. Results

3.1. Subject inclusion, hormone data and characteristics (Table 1)

During the inclusion procedure, 1000 subjects (31% recruited at psychiatric centers, and 69% through media announcements) completed telephonic screening, and 321 were invited for diagnostic interviews. Eventually, 187 patients and 72 controls were eligible to participate. This recruitment led to 1683 conceivable cortisol measures for patients and 216 for controls. Of the 187 included patients, 15 dropped out of the study’s CT treatment immediately, but we were able to collect HPA-axis data and so they were included in all analyses. Drop-outs were younger than completers, but did not differ on other characteristics (p > .05). For the 172 remaining patients, 10.7%, 21.7% and 42.6% measures were missing at T0, T1 and T2 respectively.

For the 216 conceivable values of the 72 controls, 10.6% of the measures were missing. Of the 1361 complete measures, seven values were assigned missing because of suggestive blood contamination.

Table 1: Subject characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n = 187)</th>
<th>Controls (n = 72)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, %</td>
<td>68.1</td>
<td>72.7</td>
<td>.46</td>
</tr>
<tr>
<td>Age, mean (SD), year</td>
<td>44.2 (9.7)</td>
<td>44.9 (9.3)</td>
<td>.61</td>
</tr>
<tr>
<td>Educational level a</td>
<td></td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Low, %</td>
<td>33.2</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Middle, %</td>
<td>32.6</td>
<td>19.7</td>
<td></td>
</tr>
<tr>
<td>High, %</td>
<td>34.2</td>
<td>72.3</td>
<td></td>
</tr>
<tr>
<td>Smoking, %</td>
<td>29.9</td>
<td>22.9</td>
<td>.28</td>
</tr>
<tr>
<td>Weight, mean (SD), kg</td>
<td>78.9 (16.3)</td>
<td>73.8 (13.4)</td>
<td>.04</td>
</tr>
<tr>
<td>Waist circumference, mean (SD), cm</td>
<td>89.3 (13.9)</td>
<td>83.7 (12.3)</td>
<td>.01</td>
</tr>
<tr>
<td>Hip circumference, mean (SD), cm</td>
<td>105.3 (11.1)</td>
<td>103.1 (7.8)</td>
<td>.13</td>
</tr>
<tr>
<td>Oral contraceptive use, %</td>
<td>22.1</td>
<td>17.1</td>
<td>.40</td>
</tr>
<tr>
<td>Steroid useb, %</td>
<td>.6</td>
<td>1.4</td>
<td>.57</td>
</tr>
<tr>
<td>Benzodiazepine use, %</td>
<td>8.0</td>
<td>NA</td>
<td>.57</td>
</tr>
<tr>
<td>Continuous AD use during follow-up, %</td>
<td>27.3</td>
<td>NA</td>
<td>NA</td>
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<td>Antidepressant use at study entry, %</td>
<td>42.2</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>TCA, %</td>
<td>3.9</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SSRI, %</td>
<td>29.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Other, %</td>
<td>9.1</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table is continued on the next page >
Received cognitive therapy, %  51.9  NA
HDERS17 score, mean (SD)  3.8 (2.9)  NA
Number of previous episodes, mean (SD)  6.3 (8.1)  NA
Five or more previous episodes, %  40.6  NA
Age of onset first episode, mean (SD), year  28.5 (12.5)  NA
Relapse during the 2 year follow-up period, %  54.5  NA
Depressed at T1, %  15.0  NA
Depressed at T2, %  16.0  NA
Negative early life events, %  50.3  NA
Daily hassles score T0, mean (SD)  52.5 (38.9)  NA
Daily hassles score T1, mean (SD)  41.3 (30.2)  NA
Daily hassles score T2, mean (SD)  42.2 (35.1)  NA

Abbreviations - AD = antidepressant; HDERS = Hamilton depression rating scale; SSRI = selective serotonin reuptake inhibitor; T0, T1, T2 = study entry 3 months and 2 years of follow-up respectively; TCA = tricyclic antidepressant.

b Educational level is defined as: low primary education or preparatory middle-level applied education; middle higher general continued education or middle-level applied education; and high preparatory scientific education higher applied education or scientific education.

b Steroid use in the month before assessment.

Patients and controls were successfully matched regarding age and sex. The included patient group was characterized by high recurrence rates; the mean number of previous MDEs was 6.3, and 54.5% had a recurrence during the two-year follow-up.

3.2. HPA-axis disturbance as a trait (Fig. 1)
Remitted patients had significantly higher cortisol concentrations than controls (group-effect; \( p < .001 \); adjusted for potential confounders [using PSi]). The course over the day was not significantly different between patients and controls and therefore omitted from the model (group × moment interaction; \( p = .376 \)).
Figure 1

Morning and evening cortisol concentrations for remitted recurrent MDD-patients compared to controls. All results are adjusted for sex, age, educational level, contraceptive and steroid use, smoking, weight and waist and hip circumference. Error bars indicate SE. Mixed model analyses results: remitted patients versus controls $F_{1,656.13} = 14.77$, $p < .001$.

3.3. HPA-axis disturbance as a state (Fig. 2)

The within-subject time-dependent variable modeling depressive state (current MDE during follow-up measurement) had no significant influence on cortisol concentrations (state-effect; $p = .419$), after omission of the non-significant effect on course over the day (state × moment interaction; $p = .833$). In addition, the continuous BDI-score was used to assess state-effects as well. This approach also did not reveal any significant state-effects ($p = .467$). Correction for possible confounding by antidepressant use did not change these findings.
Figure 2

Effect of a current depressive episode at sampling moment after three months (T1) and two years of follow-up (T2), on cortisol concentrations in recurrently depressed patients. Error bars indicate SE. Mixed model analyses results: being depressed according to the SCID at sampling moment (yes/no) $F_{1,65.54} = .66, p = .419$. Measures at study entry (T0) were included in the analyses, but because none of the subjects was depressed at that moment (exclusion criterion) T0 is not included in this figure.

3.4. Influence of daily hassles, CLEs and previous episodes (Fig. 3)

The interaction of daily hassle score on a given time point during follow-up (T0, T1 and T2) with moment, was not significant and therefore omitted (hassles × moment-interaction; $p = .249$). This indicates there were no associations between daily hassles and cortisol-course over the day. In the subsequent most parsimonious model there were no associations between overall cortisol concentrations and daily hassle score (hassles-effect; $p = .744$).

For CLEs, interactions with follow-up and moment were not significant, and therefore omitted from the model. In the subsequent most parsimonious model, the main-effect of CLEs also was non-significant ($p = .254$).
Cortisol concentrations in relation to the experience of childhood life events and the number of previous episodes (<5/≥5) in recurrently depressed patients during a two-year follow-up (T0, T1 and T2). All results are adjusted for sex, age, educational level, contraceptive and steroid use, smoking, weight and waist and hip circumference, alcohol and drug use (yes/no), benzodiazepine therapy (yes/no), receiving CT treatment (yes/no) and using continuous antidepressants (yes/no). Mixed model analyses results for the effect of CLEs: CLEs (yes/no) $F_{1,12.14} = 1.435, p = .254$. Mixed model analyses results for previous episodes: ≥5 previous episodes (yes/no) $F_{1,46.15} = 4.152, p = .047$. 
Regarding the number of previous episodes, there were no significant differences in cortisol-course over the day (MDEs × moment-interaction; \( p = .818 \)) or follow-up (MDEs × follow-up-interaction; \( p = .510 \)) between patients with <5 previous MDEs compared to patients with \( \geq 5 \) previous MDEs, so these interactions were omitted from the model. In the most parsimonious model the main-effect of previous MDEs was significant (MDEs; \( p = .047 \)), with lower cortisol concentrations in the patients with \( \geq 5 \) previous MDEs, compared to the patients with <5 MDEs.

3.5. Association with recurrence (Fig. 4)
There were no differences in cortisol-courses over the day or follow-up between patients who experienced a recurrence compared with those who remained in remission (recurrence × moment-interaction; \( p = .707 \), recurrence × follow-up-interaction; \( p = .957 \)). In the final, most parsimonious, model the main effect of recurrence was also non-significant (recurrence during follow-up-effect; \( p = .513 \)).

Figure 4

Cortisol concentrations in recurrently depressed patients who remained in remission during a two-year follow-up (T0, T1 and T2) compared to those who did experience at least one recurrence. All results are adjusted for sex, age, educational level, contraceptive and steroid use, smoking, weight and waist and hip circumference, alcohol and drug use (yes/no), benzodiazepine therapy (yes/no), receiving CT treatment (yes/no) and using continuous antidepressants (yes/no). Error bars indicate SE. Mixed model analyses results: experiencing a recurrence during follow-up (yes/no) \( F_{1,135.90} = .43, p = .513 \).
3.6. Effect of cognitive therapy (Fig. 5)
At T0, before CT, cortisol-course over the day was comparable between patients that were randomly assigned to receive CT and patients that were assigned to not receive CT (CT × moment-interaction; \( p = .216 \)), and therefore omitted. The main effect of CT was also non-significant (CT-group-effect \( p = .430 \)), indicating comparable cortisol concentrations in the two randomized groups before randomization to CT.

Figure 5

![Graph showing cortisol concentrations](image)

Figure 5
Effect of a randomized 8-week cognitive therapy (CT) module at study entry (T0), on cortisol concentrations after three months (T1) and two years (T2) of follow-up, in recurrently depressed patients. Error bars indicate SE. Mixed model analyses results for CT: main effect of CT (yes/no) \( F_{1,68.07} = 3.07, p = .085 \), and CT × sampling moment (morning/evening) interaction \( F_{1,18.85} = 4.30, p = .052 \).

During follow-up after CT (T1 and T2), when comparing the patients who received CT in the first eight weeks of the study with patients who did not, the 3-way-interaction between follow-up time (T1, T2), sampling moment and CT was nonsignificant (CT × follow-up moment-interaction; \( p = .960 \)), so this term was omitted. Thereafter, the interaction of CT with follow-up was also nonsignificant (CT × follow-up-interaction; \( p = .514 \)) and omitted. In the subsequent model, the interaction of CT with sampling moment reached borderline significance (CT × moment-interaction; \( p = .052 \)), indicating steeper cortisol declines over the day in patients who received CT.
4. Discussion
To our knowledge, this is the first HPA-axis study in remitted patients with recurrent MDD which applied a longitudinal repeated-measures design. With this design, we showed that high cortisol represents a trait in recurrent MDD, while there is no apparent state-effect.

First, we found significantly higher cortisol concentrations in patients compared to controls, suggestive of HPA-axis hyperactivity as a trait in recurrent MDD. Second, our data did not show state-effects on HPA-axis activity, because cortisol did not change during MDEs during follow-up. Third, HPA-axis hyperactivity was neither associated with (a) daily hassles (epiphenomenal effects), (b) CLEs (early programming), nor (c) number of previous MDEs (i.e. scarring, which on the contrary, was associated with lower cortisol concentrations). Fourth, in patients, the hypercortisolemic trait was, unexpectedly, not associated with recurrence during entire follow-up. Finally, CT caused long-lasting steeper cortisol declines over the day during the two-year followup (borderline significant).

4.1. Trait of increased cortisol
The present study corroborates with previous studies which reported higher cortisol concentrations in remitted MDD-patients 17, 3, thereby not supportive of earlier reports of HPA-axis hypoactivity 9. As distinct from previous crosssectional studies comparing HPA-axis activity in heterogeneous samples (recurrent and first episode MDD-patients combined) 17, 18, 9, our data longitudinally describe the course of the cortisol-abnormalities specifically in highly recurrent MDD-patients. Previous more heterogeneous samples might possibly have underestimated HPA-axis abnormalities 3. Our finding of persistently elevated cortisol concentrations suggests a permanent hypercortisolemic trait in this subpopulation of recurrent MDD-patients. This is further supported by studies reporting sustained higher cortisol concentrations preceding a first MDE 45, 20, 46.

4.2. Absence of state-effects
The absence of state effects in our two-year follow-up comparing different levels of depressive symptoms within subjects, extends and strengthens previous cross-sectional research which reported no differences in cortisol between currently depressed patients and remitted MDD-patients 17. The absence of state-effects may appear in contrast with previous treatment-studies, which found changes in HPA-axis activity after acute MDE treatment with antidepressants 47. Although suggestive of state-dependent changes, this influence of antidepressants could also be explained by an effect of antidepressants per se rather than a depressive state-effect 48. This is suggested by a study reporting declines in salivary cortisol concentrations during recovery from the depressive state only in responders to amitriptyline, and not in paroxetine responders 49.
In addition, because our study is the first report of longitudinal HPA-axis activity in recurrent MDD, we hope our finding of state-independency encourages further replication.

4.3. The absence of HPA-axis activating effects of daily hassles, childhood life events and previous episodes

Our data showed no association between the daily hassle scores and cortisol concentrations, rejecting the idea that increased current stress (e.g. during a MDE) has an epiphenomenal effect on the HPA-axis in recurrent MDD. Also, CLEs did not explain the observed hypercortisolemic trait, in line with previous literature. However, unexpectedly, having 5 previous MDEs was associated with lower cortisol concentrations, compared to patients with <5 previous MDEs, in our sample of recurrent MDD-patients. A possible explanation for this specific association could be that HPA-axis activity could exhaust, or get suppressed, after the experience of multiple MDEs. This could resemble parts of the effects of chronic stress on HPA-axis activity, e.g. as an adaptation to its associated increased allostatic load. Therefore, it could be hypothesized that the hypercortisolaemic trait observed in our patients is attenuated by the experience of MDEs. This could imply that the trait would have been more pronounced in our patients if we would have measured them before they experienced their MDEs.

4.4. Hypercortisolemia in recurrent MDD as an endophenotype

The absence of HPA-axis activating effects of exogenous factors in our sample, e.g. daily hassles, CLEs, and scarring effects of previous MDEs, may indicate a more endogenously regulated sustained HPA-axis hyperactivity. In addition, previous research found familial association, cosegregation, and heritability of HPA-axis activity, all criteria of an endophenotype, thereby suggestive of HPA-axis hyperactivity as an endophenotype. However, the state-independence criterion for an endophenotype, has to our knowledge not yet been addressed. With our observations of a state-independent HPA-axis hyperactivity trait, all criteria for hypercortisolemia as an endophenotype for recurrent MDD could be considered to have been fulfilled. We hope our observations will stimulate future research into the evolutionary nature of hypercortisolemia in MDD.

4.5. The association between HPA-axis activity and recurrence

We observed no association between repeatedly measured follow-up cortisol concentrations in our patients and the occurrence of recurrence(s) during this follow-up. This is consistent with studies that found no association between cortisol concentrations measured once at the start of a follow-up in long-term remitted patients and their prospective recurrence(s).
In contrast, after acute treatment, persistently high cortisol concentrations predicted recurrence during follow-up 45, 57, 58. This discrepancy could be explained by differences in study populations in the above mentioned two study-types: (I) patients who were in long-term remission (e.g. in our study >10 weeks), versus (II) acute treatment remitters. It could be hypothesized that in the latter population the patients who were not stabilized well by antidepressants had higher cortisol concentrations (e.g. by a placebo-response), and subsequent higher relapse-rates, and that these patients were responsible for the association between cortisol and relapse. This merits further exploration.

4.6. Effect of CT
In our intention to treat analysis, CT had a - borderline significant - effect of steeper cortisol declines over the day throughout the 2-year follow-up. Because baseline declines in cortisol did not differ and CT was provided at random, this effect suggests a long-lasting causal influence of CT-treatment on the hypercortisolemic trait. Interestingly, these findings could represent a biological underpinning for the recurrence preventing effect of the CT module 59. This finding corresponds with previous findings of steeper cortisol declines over the day in patients receiving psychotherapy plus antidepressants compared to patients receiving antidepressant monotherapy 60, 23. As far as we know, our data for the first time show the long-lastingness of this effect over a two-year follow-up in highly recurrent remitted MDD patients. Depending on the definition used, the effect of CT on the HPA-axis might seem in contradiction with the proposed state-independence of HPA-axis activity 29, 27. In our view, if any treatment is aimed directly at an endophenotype, such as the HPA-axis, and would be effective in the treatment of MDD-symptoms through this endophenotypic effect, it could be observed that after treatment, HPA-axis activity is diminished together with a remission of symptoms. However, in this case, it would not be a state-effect of MDD-symptoms on the HPA-axis, but an effect of CT directly at the HPA-axis, coinciding with changes in MDD-symptoms, and thereby in accordance with the state-independence criterion.

The mechanisms underlying the observed effect of CT on the HPA-axis are not elucidated yet. It could be hypothesized that the preventive CT changes coping strategies, e.g. stress perception, management of stress and generation of subsequent stress 16. These effects could possibly mediate its recurrence-preventive effects.

5. Limitations and strengths
Our study has its limitations that need to be addressed. First, because of the longitudinal design and outpatient setting, full compliance to all study protocol measurements and timing could
not be attained, resulting in potential bias from missing values. The concern of bias may be reduced by the observation that missing rates did not differ between patients and controls (10.7% vs. 10.6%, respectively). In addition, we aimed to reduce possible bias from missing values by using multiple imputation, also enabling an intention to treat analysis for the effects of CT on cortisol. Second, we only measured one morning concentration instead of the whole cortisol awakening response, and so based our cortisol course over the day estimation on two measurements. In addition, we asked subjects to provide two 0800 h saliva samples, without assessing their actual awakening times. Therefore, we do not know whether the measured morning value falls into the cortisol awakening response or not. However, all effects were estimated on these values measured systematically using identical methodology, in patients and controls and for baseline and follow-up. Conceivable consequences of these shortcomings are twofold: it could have caused differences that were actually not present, if patients handled the protocol differently than controls. On the other hand, differences could be diminished due to increases in external variability. Third, we did not include information on sleep quality in our analyses. Disrupted sleep might have influenced morning cortisol concentrations, which could have resulted in an overestimation of the difference between the patients and controls. Fourth, we did not follow-up the controls. Although this was not essential to answer our research questions, it would be very interesting to investigate stability and reactivity (e.g. in response to daily hassles) of the HPA-axis in the healthy control subjects in future research. Fifth, we were unable to correct for diet and detailed lifestyle variables, e.g. sedentary life style, employment status, sampling day (weekday vs. weekend), that possibly could have confounded results. Nevertheless, we had the opportunity to correct for smoking, alcohol/drugs use, educational level, weight, waist and hip circumference using propensity scores. These variables are associated with dietary and lifestyle characteristics and primary mediators of their effects on the HPA-axis, and therefore, the confounding possibly introduced by the lack of information on dietary and more detailed lifestyle factors is expected to be minor. Sixth, we had inadequate power to differentiate the diverse MDD-subtypes, e.g. melancholic, atypical, which could be relevant in the activity of the HPA-axis and therefore an interesting point for further research. Finally, this study was not initially set up as a strictly experimental endocrinological study. This might have resulted in smaller effect sizes. However, the naturalistic setting of our study enables follow-up of this clinical highrisk population for two years.

Our study also had major strengths. First, our inclusion procedure resulted in a well-defined patient sample, characterized by high recurrence rates, reducing the change of heterogeneity of patients and therefore inconsistent findings. This patient group is thought to (i) represent
a more biologically determined MDD-subtype, and (II) contribute largely to the major burden of MDD. Second, our longitudinal design enabled us, to our knowledge for the first time, to repeatedly sample cortisol concentrations over a two-year follow-up. This opened the possibility to determine the course of, and unravel state- and trait-effects in, HPA-axis activity.

6. Conclusion
In this longitudinal study on HPA-axis activity, we demonstrated evidence for a trait of hypercortisolemia in remitted patients with recurrent depression. Additionally, we found no state-effects on HPA-axis activity when patients became depressed again. These findings support the state-independence criterion for HPA-axis activity, and together with previous endophenotypical characteristics, the evidence for hypercortisolemia as an endophenotype for recurrent MDD is thereby further strengthened. Finally, our data indicated that preventive CT may improve the decline of cortisol over the day, which might be linked to its therapeutic effects.

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Conflict of interest
None declared.

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3.2 Lower cortisol levels predict recurrence in remitted patients with recurrent depression: a 5.5 year prospective study

Bockting CLH, Lok A, Visser I, Assies J, Koeter MW, Schene AH, The DELTA study group

Abstract

Major Depressive Disorder (MDD) is a highly recurrent disease. Stress-responsive system dysfunction seems to persist after remission. In patients with more chronic and recurrent depressive episodes, state related HPA-axis dysregulation might be a risk factor for prospective recurrence. This study examines the predictive effect of cortisol on consecutive episodes in remitted recurrently depressed patients.

Cortisol was assessed in saliva in remitted recurrently depressed patients (n=55) that were followed up prospectively for 5.5 years after remission. Recurrence was assessed using a well validated structured interview.

Lower mean morning cortisol levels predicted earlier time to recurrence over 5.5 year after correction for residual symptoms (p=0.015). Residual symptoms and childhood trauma slightly confounded the association between cortisol and recurrence. Lower cortisol levels were associated with having experienced traumatic childhood life events (42.3% in patients with lower cortisol versus 19.2% in patients with higher cortisol).

Our study provides further support for the predictive role over 5.5 year of HPA axis dysregulation, i.e. lower morning cortisol levels, of recurrence in recurrently depressed patients. Childhood trauma is associated to having lower cortisol levels. It might have long term consequences for dealing with stress and the HPA-axis.
1. Introduction
Major Depressive Disorder (MDD) is a highly recurrent disease. In the absence of prophylactic treatment, the rate of recurrence rises up to 80 percent. Therefore identifying predictors of recurrence and examining pathogenic mechanism of recurrence is essential. Stress has been considered as one of the cardinal pathogenic factors involved in MDD and its recurrence: childhood and recent life events, daily hassles, stress related to previous episodes and aberrant coping, all pose increased risks for MDD and its recurrences. Furthermore, stress reducing cognitive therapy (CT) has a beneficial effect in preventing recurrence.

The hypothalamic pituitary adrenal (HPA) axis is the major neuroendocrine stress response system. Stress-responsive system dysfunction seems to persist after remission of acute depression. The dynamics associated with the course of the illness have not been thoroughly studied yet. Persistent dysregulation of the HPA axis after remission of depression may represent a trait-marker for the risk of recurrent depressive episodes.

Mixed findings have been reported for the role of cortisol. A previous finding of our group indicated that in remitted patients with major depression, higher posttreatment maximal cortisol levels on the DEX/CRH test were associated with relapse and with shorter relapse-free survival in a mixed outpatient group with the first and recurrent episodes. In addition, the proportion of remitted patients showing a persistent DST non-suppression were suggested to be more vulnerable to early relapse and recurrence, have poor outcome after discharge and suffer more often from persistent depression. Hypercortisolism has also been reported by Zobel et al. (2001) as a marker for vulnerability for relapse and recurrence in previously depressed patients.

However, hypocortisolism has been reported as well; a phenomenon that is characterized by a hyporesponsiveness on different levels of the HPA axis in a number of stress-related states as Fries et al. stated in their overview. It was first reported in the eighties by using single-dose metyrapone test in depressed patients. Recently, Vreeburg studied in a longitudinal study including 837 patients with depressive and/or anxiety disorders, the association between salivary cortisol measures at baseline and the course of psychopathology. The patients with a lower cortisol awakening response were at a higher risk of developing a chronic course, compared to persons experiencing remission during the two-year follow up. Evening cortisol and cortisol suppression after dexamethasone intake were not associated with a chronic course. The association appeared to be similar across disorders (anxiety disorder, depressive disorder or co-morbid disorders). Another study found stable hyporeactivity of the HPA over one year in
depressed women on job-stress-related long-term sick leave compared with controls. Moreover, Sondeijker et al. reported over a study including young, not depressed adolescents, that low morning cortisol levels predicted future psychopathology.

In patients with more chronic or recurrent depressive episodes, state related HPA-axis dysregulation might be reflected in a cortisol hyposecretion when compared to non-depressed control-subjects. In a remitted recurrently depressed cohort of women, HPA system hypoactivity was found, both in the basal state and in response to a psychosocial stressor when compared to healthy subjects. In other disorders associated with chronic stress, such as posttraumatic stress disorder, cortisol hyposecretion has also been reported and for a meta-analysis see. The impact of chronic stress in recurrent depression could explain why a low HPA-Axis activity could be a risk factor for recurrence.

There have been at least 361 studies performed that compared HPA axis function between depressed and non-depressed individuals, however few studies examined the predictive value of the HPA activity on course in recurrent and chronic depression (for a meta-analysis see). The current longitudinal study examined the role of the HPA axis on prospective recurrence over 5.5 years in remitted patients with recurrent depression (i.e. having at least 2 previous episodes). All patients (N=172) achieved a good remission state at entry of the study (i.e. not meeting criteria of a depressive episode according to the DSM-IV-TR criteria and a HRSD score less than 10), though residual symptoms are common after remission in depression. Since cortisol levels are considered as rather state dependent, controlling for residual depressive symptoms in these remitted patients is necessary. We aimed to determine whether HPA-axis measures predict time to recurrence in remitted recurrently depressed patients corrected for residual depressive symptoms. In line with the previous studies on chronic recurrent depression we expect that in this highly recurrent remitted MDD group lower cortisol levels predict prospective recurrence over 5.5 year. Stress and childhood trauma might affect the predictive value of the HPA-axis on recurrence. Therefore, we will examine the role of stress (current daily hassles) and childhood trauma on the predictive value of the HPA-axis on recurrence.

2. Methods

2.1. Participants and procedure

For this study we included patients from a clinical trial in which the effect of regular care (including no care at all) on recurrence was compared to regular care with additional preventive cognitive therapy. To be eligible for the trial, subjects had to meet the following criteria: (a) at least two
Major Depressive Episodes (MDEs) in the last five years, as defined according to DSM-IV (1994) and assessed by the Structured Clinical Interview for DSM-IV (SCID, 35) by trained evaluators; (b) current remission status according to DSM-IV criteria, for longer than 10 weeks and no longer than two years ago; (c) Hamilton Rating Scale for Depression 20 of <10 (as is common in relapse/recurrence prevention studies). Exclusion criteria were current mania or hypomania or a history of bipolar illness, any psychotic disorder (current and previous), organic brain damage, alcohol or drug misuse, predominant anxiety disorder, recent ECT, recent Cognitive Therapy (CT) or receiving CT at the start of the study, or current psychotherapy with a frequency of more than two times a month. Co-morbidity on axis I was assessed using the SCID 35. There was no restriction in using pharmacotherapy (the effect of use will be examined). Participants were recruited at psychiatric centers and through media announcement. They provided informed consent to enter the protocol. The protocol was approved by the institutional ethics review committees. We were able to collect HPA-axis baseline data from 55 patients in the control group (N=84; regular care only). More detail about participants, recruitment, inclusion and exclusion criteria are available in Bockting et al. 8.

2.2. Study measures
2.2.1. Inclusion criteria and primary outcome measure
Participants were screened on inclusion and exclusion criteria via the telephone version of the Structured Clinical Interview for DSM-IV (SCID-I, 36, 35). Kappa for interrater agreement between the interviewers (psychologist/research assistants), based on audiotaped interviews, for inclusion or exclusion was 0.77, which is indicative of good/excellent agreement. Time to recurrence was also assessed with the SCID-I 35. At baseline and at five follow-up assessments (3, 12, 24, 36 and 66 months), current and past depressive episodes (covering the prior months) were checked from the start of the study. All interviews were audiotaped. Two independent experienced psychiatrists who were blind to treatment condition evaluated all participants meeting the DSM-IV criteria for major depression. In cases of disagreement, the ratings of the psychiatrists were used for further analyses. The kappa for interrater agreement between the interviewers and psychiatrist on categorization of a recurrence versus no recurrence was 0.96, indicating high agreement.

2.2.2. Prediction variables
2.2.2.1. Cortisol
Salivary cortisol. Subjects were asked at baseline to provide saliva in neutral cotton salivettes (Sarstedt AG and Co, Nümbrecht, Germany) at home at three time points on two consecutive days (08:00 and 22:00, day 1, 08:00 day 2). Saliva reliably reflects the blood cortisol concen-
trations, in a relatively stress-free and minimally intrusive way. They were instructed to rinse their mouth with water, not to brush their teeth and remain in the fasting state before collecting the sample, and to keep the samples in the refrigerator until sending the samples back to the clinic. Storage took place at -20 °C on day 3. Smoking, age, the use of antidepressants, benzodiazepines, oral contraceptives, and body mass index were recorded. Salivary cortisol was determined by radioimmunoassay (RIA) designed for saliva samples (IBL Hamburg). The intra-assay variation for cortisol was intra- and inter-assay variations were 5.1% and 6.5%, respectively of a subsample (55 of 84 patients) hormone measures that was collected. No significant differences on any of the patient characteristics and time to recurrence were detected between this sample and the complete sample ($N=84$; all $p’s >0.10$). The following hormone continued variables were calculated: (1) mean morning cortisol and (2) evening cortisol. Since the distributions of these measures were skewed transformed variables (by taking the natural logarithm) were used in the survival analyses.

### 2.2.2.2. Severity of depressive residual symptoms

The 17-item Hamilton Rating Scale for Depression (HRSD, 38) was used to assess participants’ baseline levels of depressive symptomatology (of <10). The HRSD, administered by psychologist/research assistants who were blind to treatment condition, is a widely used semi-structured clinical interview that covers a range of affective, behavioral and biological symptoms and has acceptable psychometric properties 39. Scores can range from 0 to 52. Our four interviewers second rated 17 interviews. The Intraclass Correlation (ICC) was 0.94, indicating high agreement. Since the distributions of these measures were skewed transformed variables (by taking the natural logarithm) were used in the survival analyses.

### 2.2.2.3. Stress: daily hassles and childhood trauma

To measure baseline daily hassles, the 114-item Everyday Problem Checklist was used (EPCL). The items of the EPCL refer to stressors of daily living, particularly those in the domains of work, parenthood, relationship, and household activities (continuous score). The EPCL assesses the frequency of daily hassles over the past two months, and has good psychometric properties 40.

We assessed childhood trauma covered 0-15 years (such as sexual abuse) with the 15-item Negative Life Events Questionnaire (dichotomized score) 41. This questionnaire proved to have a good predictive validity, as the number of negative life events predicted MDD-symptom severity 42.

### 2.3. Statistical analysis
The effect of cortisol on time to recurrence of a new episode was assessed with Cox regression; this takes into account differences in time at risk and censoring (no recurrence during the study period).

All analysis included the level of baseline residual symptoms (HDRS) to adjust for state effects. To examine whether the effect of HPA-measures on time to recurrence was modified by the level of residual symptoms, we first tested the interaction of cortisol (A) with residual symptoms (P); $Y = \beta_1 A + \beta_2 P + \beta_3 AP$ assesses whether residual symptoms modifies the effect of a cortisol on time to recurrence. This is the case when the coefficient of the 2-way interaction ‘cortisol by residual symptoms term’ is statistically significant. If this interaction term is not significant, $Y = \beta_1 A$ applies (which states that recurrence is related to cortisol).

To examine whether the effect of cortisol on time to recurrence was modified or confounded by current stress (daily hassles) and childhood trauma, a three-step procedure was used:

- **Step I**: Assesses whether either stress or childhood trauma (P) modifies the effect of cortisol (A) on recurrence (model $Y = \beta_1 A + \beta_2 P + \beta_3 AP$, this is the case when the coefficient of the 2-way interaction cortisol by stress or childhood trauma is statistically significant).

- **Step II**: If the 2-way interaction cortisol by stress or childhood trauma interaction term in this model is not significant, potential effect modification by either stress or childhood trauma is examined (confounding effect). Following the frequently used rule of thumb the confidence intervals of the models will be compared and are considered to be different in case they differ more than 10 percent. Effect modification will be reported.

- **Step III**: If there is no effect modification, $Y = \beta_1 A$ applies (which states that recurrence is related to cortisol).

To examine whether potential confounders (smoking, age, the use of antidepressants, benzodiazepines, oral contraceptives, and body mass index) confounded the association of cortisol with time to recurrence, we first examined whether the potential confounder itself predicted recurrence (alpha level of 0.10). Only in case of the variable predicted recurrence we examined further whether the effect of cortisol on time to recurrence was modified or confounded by the variable using the same procedure as described above. We used an alpha level of 0.05 for all survival analyses and 0.10 for interaction terms.
3. Results

Table 1 presents the characteristics of this patient group. As in most depression studies almost three quarter of the patients are women, 40.0% are single. The mean HRSD score is 4 with a median of 3 previous episodes. Childhood trauma was reported by 30.2% of the sample. Not all remitted patients receive treatment (34.5%), whereas 58.2% use antidepressant medication (58.8% of them use SSRI’s). Benzodiazepine was used by 1 patient and no menopausal hormone treatment was reported. In this sample 29.1% used oral contraceptives.

Table 1: Demographic and clinical characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>(N=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, female (%)</td>
<td>72.7</td>
</tr>
<tr>
<td>White (%)</td>
<td>98.0</td>
</tr>
<tr>
<td>Age (yr, mean±S.D.)</td>
<td>43.8±9.5</td>
</tr>
<tr>
<td>Years of education (mean±S.D.)</td>
<td>14.2±2.5</td>
</tr>
<tr>
<td>Single (%)</td>
<td>40.0</td>
</tr>
<tr>
<td>Type of current treatment (%)</td>
<td></td>
</tr>
<tr>
<td>Family doctor</td>
<td>25.5</td>
</tr>
<tr>
<td>Psychiatric help</td>
<td>40.0</td>
</tr>
<tr>
<td>No treatment</td>
<td>34.5</td>
</tr>
<tr>
<td>Antidepressant medication (%)a</td>
<td>58.2</td>
</tr>
<tr>
<td>SSRI (%)</td>
<td>58.8</td>
</tr>
<tr>
<td>SNRI (%)</td>
<td>17.7</td>
</tr>
<tr>
<td>TCA (%)</td>
<td>5.9</td>
</tr>
<tr>
<td>Other (%)</td>
<td>11.8</td>
</tr>
<tr>
<td>HRSD-17 score (mean±S.D.)</td>
<td>3.5±29</td>
</tr>
<tr>
<td>Median previous episodes±IQRb</td>
<td>3 (2-6)</td>
</tr>
<tr>
<td>Age of first onset (yr, mean±S.D.)</td>
<td>27.8±13.0</td>
</tr>
<tr>
<td>Co-morbid anxiety disorder (%)</td>
<td>10.9</td>
</tr>
<tr>
<td>Posttraumatic stress disorder (%)</td>
<td>0</td>
</tr>
<tr>
<td>Reported a childhood traumatic events (%)c</td>
<td>30.2</td>
</tr>
<tr>
<td>Reported general life events before 16 (%)</td>
<td>81.8</td>
</tr>
<tr>
<td>Daily hassles (geometric mean±SE)d</td>
<td>3.26±.09</td>
</tr>
<tr>
<td>Oral contraceptives (%)</td>
<td>29.1</td>
</tr>
<tr>
<td>Benzodiazepines (%)</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table is continued on the next page >
There were no ‘non-detectables’ on cortisol. The detection limit was 0.40 nmol/l. Mean morning cortisol over 2 day was 20.06 nmol/l (S.D.=9.61). Mean evening cortisol over 1 day was 3.14 nmol/l (S.D.=3.46). For the analyses log transformed data were used because of skewed data. Morning cortisol (ln) on day one was highly correlated to morning cortisol (ln) on day two (Pearson correlation 0.609, \( p < 0.001 \)). There was no difference between men and women on baseline mean cortisol levels (ln): \( t(53) = 0.008, p = 0.99 \) (Men: \( M = 2.88, S.D. = 0.62 \), Women: \( M = 2.88, S.D. = 0.46 \)). Baseline mean morning cortisol (ln) levels differed between baseline antidepressant users versus non-users, indicating lower baseline levels of mean morning cortisol in patients that use antidepressants: \( t(53) = 52.334, p = 0.029 \) (AD users: \( M = 2.77, S.D. = 0.57 \), Non-users: \( M = 3.04, S.D. = 0.36 \)). There was no significant difference between baseline evening cortisol (ln) levels and antidepressant users versus non-users, though there was a difference on the level of a trend, indicating lower baseline levels of evening cortisol in patients that use antidepressants: \( t(53) = 1.973, p = 0.054 \) (AD users: \( M = 0.65, S.D. = 0.71 \), Non-users: \( M = 1.05, S.D. = 0.77 \)).

Relapse/recurrence

Over the 5.5 years follow-up period, 43 (78%) of our 55 participants were diagnosed with a new depressive episode. The mean time to recurrence was 668 day (Standard Error=95.96) with a median of 390 day (range: 242.39-537.61).

Comparison patients with and without a prospective recurrence

A comparison between relapsed patients versus non-relapsed patients over the 5.5 year period revealed no differences between the two groups (for evening cortisol: \( t(53) = 0.564, p = 0.575 \); for patients with a recurrence, evening cortisol (ln) \( M = 0.8473, S.D. = 0.7908 \); for patients without a recurrence, \( M = 0.7073, S.D. = 0.6362 \); for HRSD score: \( t(53) = 1.407, p = 0.167 \); for patients with a recurrence, mean HRSD score (ln) \( M = 2.50, S.D. = 2.97 \); for patients without a recurrence,
$M=3.79$, S.D.$=2.78$, except for baseline mean morning cortisol levels (ln) at the level of a trend, indicating lower baseline levels of mean morning cortisol in patients that relapsed ($t(53)=1.940$, $p=0.058$; for patients with a recurrence, mean morning cortisol (ln) $M=2.814$, S.D.$=0.4891$; for patients without a recurrence, $M=3.126$, S.D.$=0.5058$).

3.1. Prediction of time to recurrence by cortisol

Mean morning cortisol predicted time to recurrence after correction for residual depressive symptoms (after correction for residual depressive symptoms: $Wald(1, N=55)=5.889$, $p=0.015$, Hazard ratio$=0.442$, 95% CI$=0.228-0.855$, without including residual symptoms: $Wald(1, N=55)=3.374$, $p=0.066$, Hazard ratio$=0.554$, 95% CI$=0.295-1.040$). Lower mean morning cortisol levels predicted earlier recurrence. Evening cortisol did not predict time to recurrence (after correction for residual depressive symptoms: $Wald(1, N=55)=0.094$, $p=0.759$, Hazard ratio$=0.938$, 95% CI$=0.623-1.412$, without including residual symptoms: $Wald(1, N=55)=0.052$, $p=0.820$, Hazard ratio$=0.956$, 95% CI$=0.648-1.409$). Both interaction terms (i.e. mean morning cortisol by residual symptoms and evening cortisol×residual symptoms) did not predict time to recurrence (both $p’s>1$).

As presented in Table 2 none of the potential confounders (i.e. anti-depressant use, use of oral contraceptives smoking, BMI and age) was associated to time to recurrence. So we detected no moderation or confounding effect on the association between mean morning cortisol and recurrence.

Table 2. Potential confounders of the association between mean morning cortisol and recurrence* ($N=55$)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Patients ($n=187$)</th>
<th>Controls ($n=72$)</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate prediction of relapse</td>
<td>Mean morning cortisol</td>
<td>Anti-depressant use</td>
<td>Contraceptive use</td>
</tr>
<tr>
<td>Wald</td>
<td>5.889</td>
<td>0.018</td>
<td>1.723</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$-0.817$</td>
<td>$-0.042$</td>
<td>$-0.428$</td>
</tr>
<tr>
<td>$\text{Exp}(\beta)$</td>
<td>0.442</td>
<td>0.59</td>
<td>0.652</td>
</tr>
<tr>
<td>$P$</td>
<td>0.015</td>
<td>0.892</td>
<td>0.189</td>
</tr>
<tr>
<td>CI</td>
<td>$0.228$-$0.855$</td>
<td>$0.522$-$1.762$</td>
<td>$0.344$-$1.235$</td>
</tr>
</tbody>
</table>

* All models corrected for residual symptoms.

* Continuous variables: mean morning cortisol, residual symptoms, age, BMI (all log transformed because of skewness), all the other variables were dichotomous.
3.2. Association with daily hassles and childhood life events and traumatic childhood events

Mean morning cortisol is correlated at the level of a trend with having traumatic experienced life events before the sixteenth year (mean morning cortisol (ln) dichotomized using a median split; Kappa = 0.231, p = 0.071). In the group with lower cortisol, 42.3% (11/26) experienced traumatic life events before age 16, while for the group with higher cortisol this was 19.2% (5/26).

As shown in Table 3 the number of daily hassles did not modify the association between recurrence and mean morning cortisol corrected for residual symptoms (interaction term: Wald(1, N=55) = 2.276, p = 0.131, Hazard ratio = 0.417, 95% CI = 0.134-1.299). In addition, daily hassles did not confound the association between lower mean morning cortisol and earlier recurrence (adjusted hazard for cortisol; Wald(1, N=55) = 6.546, p = 0.011, Hazard ratio = 0.418, 95% CI = 0.214-0.815).

As shown in Table 3 (n=3 missing), having experienced traumatic events in childhood (for example sexual abuse) did not modify the association between recurrence and mean morning cortisol corrected for residual symptoms (interaction term: Wald(1, N=52) = 1.77, p = 0.674, Hazard ratio = 1.165, 95% CI = 0.571-2.376). It did however confound this association (>10% change of confidence intervals of cortisol including childhood trauma in the model): including childhood trauma Wald(1, N=52) = 3.641, p = .056, Hazard ratio = 0.512, 95% CI = 0.258-1.018 versus 95% CI without childhood trauma: Wald(1, N=52) = 5.789, p = 0.016, Hazard ratio = 0.439, 95% CI = 0.224-0.858. An exploratory multivariate analysis including mean morning cortisol, daily hassles and childhood trauma (after correction for residual symptoms) revealed that all variables independently (interaction terms could not be tested) predict time to recurrence (n=52, n=3 is missing: for total model p = 0.001, for mean morning cortisol p = 0.051, for childhood trauma, p = 0.024 and for daily hassles p = 0.055).
Table 3. Role of stress and childhood trauma on the association between mean morning cortisol and recurrence (N=55)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potential confounder by mean morning cortisol interaction</td>
<td>Potential confounder</td>
<td>Mean morning cortisol</td>
</tr>
<tr>
<td>Daily hassles</td>
<td>Wald: 2.276</td>
<td>3.748</td>
<td>6.546</td>
</tr>
<tr>
<td></td>
<td>$\beta$: $-0.874$</td>
<td>0.466</td>
<td>$-0.872$</td>
</tr>
<tr>
<td></td>
<td>Exp($\beta$): 0.417</td>
<td>1.594</td>
<td>0.418</td>
</tr>
<tr>
<td></td>
<td>$P$: 0.131</td>
<td>0.053</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>CI: 0.134-1.299</td>
<td>0.994-2.555</td>
<td>0.214-0.815</td>
</tr>
<tr>
<td>Childhood traumatic events&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Wald: 0.177</td>
<td>4.939</td>
<td>3.641</td>
</tr>
<tr>
<td></td>
<td>$\beta$: 0.153</td>
<td>0.392</td>
<td>$-0.669$</td>
</tr>
<tr>
<td></td>
<td>Exp($\beta$): 1.165</td>
<td>1.480</td>
<td>0.512</td>
</tr>
<tr>
<td></td>
<td>$P$: 0.674</td>
<td>0.026</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>CI: 0.571-2.376</td>
<td>1.047-2.091</td>
<td>0.258-1.018&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Continuous variables: mean morning cortisol, residual symptoms, daily hassles (all log transformed because of skewness), dichotomous: Childhood trauma.

<sup>b</sup> Model I: ln mean morning cortisol by potential confounder interaction, corrected for residual symptoms.

Model II: ln mean morning cortisol, potential confounder corrected for residual symptoms.

Model III: ln mean morning cortisol corrected for residual symptoms.

<sup>*</sup> n=3 missing.

<sup>**</sup> Confounding effect for traumatic childhood events trauma.

4. Discussion

We examined the predictive value of HPA-axis over 5.5 years in patients with remitted recurrent depression. In line with our expectation we found that relatively lower mean morning cortisol levels predicted within this patient group, earlier time to recurrence over this 5.5-year period, while evening cortisol did not predict time recurrence. A multivariate analyses revealed that mean morning cortisol, childhood trauma and daily hassles tend to be independently associated to recurrence. However, interaction terms in this multivariate model could not be tested given the relatively small sample (n=55). Though childhood trauma slightly confounded the prediction of recurrence by mean morning cortisol. This supports prior findings that chronicity and recurrence in depression is associated with lower levels of cortisol. Ahrens et al. reported hypoactivity over a remitted recurrently depressed cohort with exclusively women. We found no significant differences in mean morning cortisol levels between men and women.
The impact of stress in recurrent depression could explain why a relatively low HPA-Axis activity is a risk factor for recurrence in recurrent depression. In recurrent depression daily hassles and life events seem to predict subsequent recurrence. Daily hassles and life events may result in states of chronic stress exposure which may cause an allostatic load, i.e., fluctuation and heightened neural or endocrine response, as pointed out by Fava.

Having experienced traumatic events in childhood might have a crucial role in the HPA-axis, in dealing with stress and subsequently in onset and course of depression (chronicity). Childhood traumas have been reported before as an independent determinant of chronicity of depression. This is in line with our finding that lower cortisol levels within this patient group were associated to having experienced more traumatic life events in childhood and predicted prospective recurrence.

Carpenter and colleagues reported that especially emotional childhood abuse might dampen cortisol reactivity that is cumulative overtime, which has been shown in a study amongst 230 adults without major Axis I Disorders that completed the Dex/CRH test. Unfortunately, we do not have data on emotional abuse in childhood to examine this hypothesis. However, we did indeed find an indication that childhood trauma in general (such as sexual abuse) slightly confounded the prediction of recurrence by mean morning cortisol.

Miller et al. showed in their meta-analysis on the impact of chronic stress on HPA function that much of the variability in HPA response is attributable to stressor features. Timing is a critical element, as hormonal activity is elevated at stress onset but reduced as time passes. Stress that threatens physical integrity is traumatic in nature, and is largely uncontrollable that elicits a high, flat diurnal profile of cortisol secretion. Together, this can result in poor regulation of stress, and could play a role in both the initiation of depression and increased vulnerability to recurrence. Alternatively, lowered cortisol levels in the remitted state may reflect a direct biological vulnerability for recurrent depressive episodes which, consequently, heightens the impact of daily hassles in these patients. To explain our findings, one might hypothesize that during the course of recurrent depression, an initial HPA overdrive may lead to increased basal cortisol levels associated with an abrupt stress response, and after remission of depression, may be followed by a dampened/muted basal as well as stress-induced HPA activity.
Alternatively, our findings if replicated might indicate the hypothetical existence of different depression phenotypes. One type may be characterized by normalization of HPA system function or even by evolving HPA hypo-activity associated with stable remission. Another type may show sustained HPA system dysfunction after remission as well as an increased risk for recurrence. One might speculate that our group of remitted patients included more subjects from the second group, giving insight in stress system regulation in this subtype.

Besides antidepressant medication, psychological interventions in the maintenance phase, such as brief cognitive therapy either added to regular care or medication, and Mindfulness-Based Cognitive therapy, are helpful in preventing relapse/recurrence. As previously reported in patients who received additional preventive cognitive therapy, cortisol levels did not predict time to relapse. Possibly in patients who were treated with psychological preventive interventions, the impact of daily hassles on recurrence is reduced, and therefore the extent to which patterns of depressive thinking were reactivated is limited, resulting in the absence of lowered cortisol as a risk factor for recurrence.

This study contains strengths and weaknesses. Strengths of the study are the fact that our cohort included exclusively patients with at least two previous episodes and was followed prospectively for 5.5 years. Further, we included patients with recurrent depression remitted on variety of treatments or no treatment at all, without restrictions on medication status at entry to the study. As such, this study was designed to maximize external validity, which suggests good generalizability of the findings. However, there are also several limitations to address. First, the relatively small sample size reducing the power to detect weaker associations between recurrence and the interaction between cortisol, daily stress and experienced childhood trauma. However, longitudinal studies in this high risk group for recurrence (i.e. patients with recurrent depression) are scarce. Second, we have no data on waking time and sleep quality of the subjects. Third, we only measured one morning concentration (mean of 2 day morning assessments) instead of the whole cortisol awakening response, and so based our cortisol course over the day estimation on two measurements. However, all our effects were estimated on values measured using identical methodology. Fourth, our results restrict to prediction of recurrence within this sample and does not include a comparison to a normal control group. Fifth, this sample included a mixed group of patients that used antidepressants and patients that did not use this. In line with other studies, we found an association between use of antidepressants and decreased cortisol levels. However, in line with Vreeburg et al.’s findings were comparable after controlling for the confounding effect of use of antidepressants. Finally, we assessed
childhood trauma by using a self-report questionnaire rather than an interview. This might underscore the actual prevalence of childhood trauma.

In sum, our study adds further support for the predictive role of HPA axis dysregulation, i.e. lower morning cortisol levels, in relapse and recurrence of recurrent depression. This effect could not be explained by state dependent depressive residual symptoms, though residual symptoms slightly confounded the association.

Childhood traumatic life events might have an impact on the HPA-axis and thereby on the impact of stress as risk factor for recurrence in depression. Replication is necessary to confirm the impact of childhood traumatic life events on the HPA-axis and thereby on prospective recurrence. Future studies are needed to examine this specific pathogenic role of cortisol in interaction with stress and childhood trauma as risk factor for recurrence in larger longitudinal samples in this chronic highly recurrent disease.

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Chapter 4
Fatty acid metabolism
Fatty acid metabolism

4.1 Fatty acids and homocysteine levels in patients with recurrent depression: an explorative pilot study

Assies J, Lok A, Bockting CLH, Weverling GJ, Lieverse R, Visser I, Abeling NGGM, Duran M, Schene AH,

Abstract
Major depressive disorders (MDD) and cardiovascular disease are mutually associated. They share signs and symptoms of the “metabolic syndrome”. Two observations that may be causally related with the metabolic syndrome and therefore with both MDD and cardiovascular disease are a decrease in ω-3 polyunsaturated fatty acids (PUFAs) and a rise in plasma homocysteine (tHcy) levels. Both the rise in tHcy and the decrease in ω-3 PUFAs may be associated with enhanced lipid peroxidation.

We exploratively studied 44 randomly chosen patients out of a cohort of 134 patients with the recurrent form of MDD (MDD-R). We measured tHcy levels together with saturated FAs, monounsaturated fatty acids (MUFAs) and PUFAs of the ω-3, ω-6 and ω-9 series in plasma and erythrocytes. Levels were compared with laboratory reference values. The main findings were a decrease in the erythrocytes of C22:5ω-3, C22:6ω-3, C24:1ω-9 and C20:3ω-9 and in the plasma a decrease in C24:1ω-9 and C20:3ω-9. The only significant association we found was between the total of ω-6 fatty acids and plasma tHcy. The FA alterations were found in patients although most of them were clinically recovered, suggesting that the alterations may represent a biological” trait” marker for recurrent depression.
1. Introduction
It is now well established that major depressive disorder (MDD) and in particular its recurrent, chronic form, recurrent major depressive disorder (MDD-R), is a growing worldwide problem. The World Health Organization (WHO) recently ranked all major medical disorders in the world by a new standardized measure of burden, the disability adjusted life years (DALY). By this measure MDD was calculated as being the fourth most disabling. One of the reasons for the great contribution of MDD to the total burden of disease is its long duration and its highly recurrent nature, with almost fifty percent of patients going into recurrence.

It has also been well documented that MDD increases the risk of cardiovascular disease and vice versa patients with cardiovascular disease suffer from depression more frequently than the general population. The precise mechanisms underlying the heart disease-depression relationship still remain to be elucidated. Patients with depression and cardiovascular disease share components of the “metabolic syndrome” (syn. syndrome X, insulin resistance syndrome). This syndrome includes a cluster of risk factors for atherosclerosis such as (visceral) obesity, hypertension, glucose intolerance, diabetes mellitus, dyslipidemia, hypercortisolemia and low grade inflammation, with insulin resistance being the common feature and basic abnormality.

Two observations that may be causally related with the metabolic syndrome and therefore with both depressive disorders and cardiovascular disease are a decrease in ω-3 fatty acids and a rise in homocysteine levels.

Fatty acid research in schizophrenia as well in major depressive disorders has yielded substantial evidence for perturbed membrane phospholipid metabolism. Cell membranes consist of phospholipid bilayers with fatty acids of different length and degree of unsaturation. Fatty acids (FAs) can be classified into three families: saturated FAs, mono-unsaturated FAs (MUFAs) and poly-unsaturated FAs (PUFAs). The major series of mammalian FAs are the ω-9, considered non-essential, and the ω-3 and ω-6 essential FAs. The latter cannot be synthesized by mammals and therefore have to be obtained from the diet. PUFAs are the products of successive desaturations and elongations of their 18-carbon chain precursors: α-linolenic acid (ALA, C18:3ω-3), linoleic acid (LA, C18:2ω-6), and oleic acid (C18:1ω-9). Unsaturated FAs in particular determine membrane fluidity and, therefore important membrane functions such as electrical signalling, receptor sensitivity, and neurotransmitter release. Plasma levels reflect dietary intake of the past fortnight rather than long-term dietary impact, which is better reflected in the erythrocyte membrane composition.
It has been postulated that the incidence in ischemic heart disease as well as depressive disorders in the past century is related to the changes in the dietary habits of Western societies which are characterized by an increased intake of total fat, in particular saturated fats and by a shift from ω-3 to ω-6 PUFAs.\(^\text{11,12}\)

In patients with major depressive disorder a significant reduction of total ω-3 PUFAs, as well as of the single FAs EPA (eicosapentaenoic acid, C20:5ω-3), DHA (docosahexaenoic acid, C22:6ω-3) and high total ω-6/ total ω-3 and high AA (arachidonic acid, C20:4ω-6)/EPA ratios were found in plasma and erythrocyte cell membranes compared with healthy controls.\(^\text{13,14,15,16,17}\) Results in ω-6 PUFAs are less consistent: no difference and a smaller sized reduction in these fatty acids than in ω-3 PUFAs were reported. Less attention was paid to the saturated FAs and MUFAs, but increases in C16:0, C18:0 and C18:ω-9 were reported.\(^\text{14,15}\)

In addition to a decreased dietary intake, an enhanced ω-3 fatty acid peroxidation may also affect the fatty acid composition. Both patients with major depressive disorder and patients with coronary artery disease showed a similar increase in lipid peroxidation compared with healthy controls.\(^\text{18}\) Lipid peroxidation is thought to play a fundamental role in the pathogenesis of atherosclerotic vascular disease.\(^\text{19}\)

Homocysteine is a sulphur-containing amino acid metabolized by two different pathways: remethylation and transsulfuration. In the remethylation pathway, which requires folic acid and vitamin B\(_{12}\) as cofactors homocysteine is converted to S-adenosylmethionine, a universal methyl donor. In the transsulfuration reaction which requires vitamin B\(_{6}\) as a cofactor homocysteine is converted to glutathione, a major intracellular antioxidant.\(^\text{20}\)

Homocysteine itself can generate reactive oxygen species (ROS) and induce lipid peroxidation (LPO).\(^\text{21}\) Total plasma homocysteine (tHcy) levels above 9-10 μmol/l are thought to represent a graded independent risk factor for arteriosclerotic vascular diseases.\(^\text{22}\)

Mean tHcy were reported to be higher in patients with major depressive disorder than in healthy controls. In one study 20% of depressed patients had tHcy levels >13.2 μmol/l, whereas in another study 50% of the depressed patients had levels >12 μmol/l suggesting that 20-50% of the patients may be at increased risk of cardiovascular morbidity (homocysteine concentrations >9 μmol/l)\(^\text{22,23,24}\).
Animal and human studies suggest a link between hyperhomocysteinemia, lipid peroxidation and a decrease in $\omega$-3 FAs. A folic-acid deficient diet increased tHcy in rats fourfold. It further induced an increase in LPO products while erythrocytes became enhanced susceptible to free radicals in vitro. At the same time there was a decrease in total $\omega$-3 FAs in plasma and a decrease in $\text{ALA}$ and $\text{DHA}$ in plasma and thrombocytes: $\text{AA}$ was increased, as was the $\text{AA}/\text{EPA}$ quotient 25. In another study the administration of folic acid significantly increased $\text{DHA}$ in rat erythrocyte phospholipids without significant changes in the $\omega$-6 FA status 26. In the plasma of pregnant women with signs of placental vasculopathy tHcy levels were elevated and negatively correlated with erythrocyte phospholipid $\text{DHA}$ of their offspring 27. Together these data suggest that enhanced tHcy levels are associated with increased LPO and low plasma and tissue $\omega$-3 FAs.

Because biological defect(s) may not only exist during depression but may also persist during the interepisodic phase of the recurrent form of MDD 28 it is relevant to further explore the relationship between homocysteine and fatty acids in patients with this recurrent type of depression. We therefore measured tHcy levels together with saturated FAs, MUFAs and PUFAs of the $\omega$-3 and $\omega$-6 and $\omega$-9 series in plasma and erythrocytes.

2. Subjects and methods
2.1. Subjects
So far we analysed plasma and erythrocytes of 44 patients, randomly chosen of a cohort of 136 patients with a high risk of recurrent depression ($\geq$2 episodes in the past). These patients participated in a randomized clinical trial comparing the efficacy of a preventive cognitive therapy with care as usual with a follow-up of 2 years.

The inclusion criteria of the main study were age between 18 and 65 years, at least two depressive episodes in (the previous) 5 years, having reached current remission status according to criteria of the fourth edition of the Diagnostic and Statistic Manual (DSM-IV), for longer than 10 weeks and no longer than 2 years ago and a current score on the Hamilton Rating Scale for depression of $<10$. Exclusion criteria were: current mania or hypomania, history of bipolar illness, psychotic disorders (current or previous), organic brain syndrome, alcohol or drug abuse, predominant anxiety disorders, recent Electro Convulsive Therapy (ECT), recent cognitive treatment, other cognitive therapy in aftercare, current psychotherapy with a frequency more than two times a month.
Time after Time; biological factors in the course of recurrent depression

The Medical Ethical Committee of the Academic Medical Centre in Amsterdam approved the study protocol. All participants gave written informed consent prior to enrollment.

2.2. Assessment of depression and other variables
At the 2-years follow up assessment of the main study, patients psychiatric status was again assessed with the Structured Clinical Interview of DSM-IV disorders (SCID-I) by trained interviewers, so patients were either relapsed (depressed) or remitted (non-depressed).

Blood was also sampled at this moment.

Information about the use of antidepressants during sampling was obtained by the Trimbos/IMTA questionnaire for Costs associated with Psychiatric Illness self report questionnaire (TIC-P:40) and by interview.

Anthropometric measurements (body mass index (kg/m2), waist circumference (WC) (cm) and waist hip ratio) were obtained during sampling time.

2.3. Blood sample collection and analysis of homocysteine and fatty acids
Venous blood samples were collected at the patients home. Plasma was separated within 4 h of collection and stored at −80°C until analysis. In all 136 patients folic acid, vitamin status and homocysteine were analysed.

The tHcy was determined with isocratic high-performance liquid chromatography (HLPC) electrospray tandem massspectrometry (MS-MS). The intra- and interassay coefficients of variation, linear in range from 2 to 150 µmol/l, were within 3.6% and 4%, respectively.

Plasma folate and vitamins B₆ and B₁₂ were measured by routine laboratory methods.

Fatty acids in plasma or erythrocytes were analysed in the random sample of 44 patients by capillary gas chromatography as their methyl esters. A 50 µl sample was added to 1 ml of a 3 M methanolic HCl solution and the lipids were hydrolysed at 90°C for 4 h, achieving simultaneous methylation of the liberated fatty acids. After cooling the fatty acid methyl esters were extracted with 2 ml hexane.
Following evaporation of the solvent, the fatty acid methyl esters were separated on a capillary free fatty acid phase (FFAP) column. All concentrations were calculated with reference to the internal standard 18-methylnonadecanoic acid.

2.4. Statistical analysis
All data were screened for distribution of normality and in case of skewed distribution variables were log transformed and geometric means were obtained and 95% confidence intervals were calculated.

In order to evaluate whether FAs of the study sample were outside the normal limits, FA means and their 95% CI of the patients were compared to in house laboratory reference values. These reference values are derived from healthy laboratory personnel (n=40) in the age range from 20 to 50 years.

Linear regression analyses were performed in order to investigate the association between levels of tHcy and the following FAs: C18:3ω-3, C20:5ω-3, C22:6ω-3, C18:2ω-6, C20:4ω-6, C18:1ω-9, C24:1ω-9, C20:3ω-9, C16:0, C18:0, C20:0, C22:0, C24:0 and the summations of the various series in erythrocytes and plasma. In this approach adjustment was made for gender and use of antidepressants. The selected FAs constitute the precursors and their respective desaturation and elongation products.

Logistic regression was applied to investigate the association between FA levels (concentrations dichotomized into above and below the median values) and depression-status (depressed/non-depressed). To adjust for multiple testing, P-values were calculated according to the method described by Holm 29.

Statistical differences were considered significant at P-values <0.05 (two-sided). All analyses were performed using the statistical package SPSS 10 for Windows (release 10.0.7, SPSS Inc, Chicago, IL).

3. Results
3.1. Characteristics of the study population
Table 1 shows the characteristics of the study sample (n=44) as compared to the cohort (n=136). The two groups were comparable with respect to: age, gender, proportion of patients with current depression, and proportion of patients using antidepressants. There were also no differences regarding BMI, WC, folic acid, vitamin B₆ and B₁₂ and tHcy.
The mean tHcy was 10.5 μmol/l (range: 7-15, SD=2.8) for men and 8.7 μmol/l (range: 6-14, SD=1.9) for women. Plasma folate and vitamin B₆ and B₁₂ were within normal limits.

### Table 1: Characteristics of patients

<table>
<thead>
<tr>
<th></th>
<th>Study sample</th>
<th>Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>44</td>
<td>136</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45±9</td>
<td>45±10</td>
</tr>
<tr>
<td>Male (%)</td>
<td>10 (23%)</td>
<td>35 (26%)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.4±5.3</td>
<td>26.9±5.2</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>86.9±13.8</td>
<td>89.3±13.9</td>
</tr>
<tr>
<td>Folic Acid (nmol/l)</td>
<td>22.6±6.8</td>
<td>23.8±7.3</td>
</tr>
<tr>
<td>Vitamin B₆ (nmol/median range)</td>
<td>89.5±87.1</td>
<td>98.5±88.4</td>
</tr>
<tr>
<td>Vitamin B₁₂ (pmol/l)</td>
<td>324.4±115.9</td>
<td>313.8±127.4</td>
</tr>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>9.1±2.2</td>
<td>9.7±3.0</td>
</tr>
<tr>
<td>Current depression (%)</td>
<td>8 (18%)</td>
<td>25 (18%)</td>
</tr>
<tr>
<td>Use of antidepressants (%)</td>
<td>29 (66%)</td>
<td>81 (60%)*</td>
</tr>
</tbody>
</table>

**Abbreviations** - BMI = body mass index.

Values are means±SD or number with percentages.

* Of 4 patients data were missing. Reference values: Folic acid 7-39 nmol/l, vitamin B₆ 35-107 nmol/l, vitamin B₁₂ 150-700 pmol/l, homocysteine male 8-18 μmol/l, homocysteine female 6-19 μmol/l.

### 3.2. Fatty acids concentrations

#### 3.2.1. Erythrocytes

We found the following erythrocyte FAs concentrations being below the lower limit of the normal reference range (see Table 2):

- In the ω-3 series: C22:5ω-3 and C22:6ω-3 (DHA)
- In the ω-6 series: C18:3ω-6 and C22:4ω-6
- In the ω-9 series: C20:3ω-9 and C24:1ω-9

Saturated FA levels: C22:0 and C24:0.

The sum of the FAs and the ratios of C22:5ω-6/C22:4ω-6 and C22:5ω-6/C22:6ω-3 were within the normal range.
Table 2: Erythrocyte fatty acid concentrations (pmol/10^6 erythrocytes)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>CI 95%</th>
<th>RV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ω-3 Series</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linolenic acid (18:3ω-3)</td>
<td>0.8</td>
<td>0.7-0.9</td>
<td>0.1-1.8</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (20:5ω-3)</td>
<td>3.2</td>
<td>2.8-3.5</td>
<td>1.1-7.7</td>
</tr>
<tr>
<td>Docosapentaenoic acid (22:5ω-3)</td>
<td>6.8ᵃ</td>
<td>6.5-7.2</td>
<td>7.6-23</td>
</tr>
<tr>
<td>Docosahexaenoic acid (22:6ω-3)</td>
<td>12.6ᵃ</td>
<td>11.7-13.4</td>
<td>15.2-37.6</td>
</tr>
<tr>
<td><strong>ω-6 Series</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (18:2ω-6)</td>
<td>76.8</td>
<td>72.9-81.0</td>
<td>52.0-89.0</td>
</tr>
<tr>
<td>Gamma-Linolenic acid (18:3ω-6)</td>
<td>0.5ᵃ</td>
<td>0.5-0.6</td>
<td>0.0-0.4</td>
</tr>
<tr>
<td>Eicosadienoic acid (20:2ω-6)</td>
<td>1.2</td>
<td>1.1-1.3</td>
<td>0.2-2.4</td>
</tr>
<tr>
<td>Homogamma-Linolenic acid (20:3ω-6)</td>
<td>8.8</td>
<td>8.1-9.6</td>
<td>6.0-19.6</td>
</tr>
<tr>
<td>Arachidonic acid (20:4ω-6)</td>
<td>71.3</td>
<td>69.2-73.4</td>
<td>67.0-107.0</td>
</tr>
<tr>
<td>Docosadienoic acid (22:2ω-6)</td>
<td>0.7</td>
<td>0.6-0.7</td>
<td>0.0-4.0</td>
</tr>
<tr>
<td>Docosatetraenoic acid (22:4ω-6)</td>
<td>8.7ᵃ</td>
<td>8.1-9.3</td>
<td>9.5-26.6</td>
</tr>
<tr>
<td>Docosapentaenoic acid (22:5ω-6)</td>
<td>1.5</td>
<td>1.3-1.6</td>
<td>1.4-6.2</td>
</tr>
<tr>
<td><strong>ω-9 Series</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypogeic acid (16:1ω-9)</td>
<td>0.9</td>
<td>0.8-1.0</td>
<td>0.0-7.2</td>
</tr>
<tr>
<td>Oleic acid (18:1ω-9)</td>
<td>83.1</td>
<td>80.7-85.4</td>
<td>58.0-115.0</td>
</tr>
<tr>
<td>Gondoic acid (20:1ω-9)</td>
<td>1.0</td>
<td>0.9-1.0</td>
<td>0.0-3.5</td>
</tr>
<tr>
<td>Erucic acid (22:1ω-9)</td>
<td>Nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nervonic acid (24:1ω-9)</td>
<td>9.5ᵃ</td>
<td>9.2-9.8</td>
<td>15.5-35.8</td>
</tr>
<tr>
<td>Eicosatrienoic acid (20:3ω-9)</td>
<td>0.3ᵃ</td>
<td>0.3-0.4</td>
<td>1.0-4.1</td>
</tr>
<tr>
<td><strong>Saturates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristic acid (C14:0)</td>
<td>3.7</td>
<td>3.4-3.9</td>
<td>0.7-6.9</td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>187.4</td>
<td>183.3-191.5</td>
<td>113.0-193.0</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>110.2</td>
<td>107.4-113.0</td>
<td>81.0-141.0</td>
</tr>
<tr>
<td>Arachidic acid (C20:0)</td>
<td>2.6</td>
<td>2.5-2.7</td>
<td>2.0-3.4</td>
</tr>
<tr>
<td>Behenic acid (C22:0)</td>
<td>6.0ᵃ</td>
<td>5.8-6.2</td>
<td>6.3-13.7</td>
</tr>
<tr>
<td>Lignoceric acid (C24:0)</td>
<td>10.1ᵃ</td>
<td>9.7-10.4</td>
<td>16.3-33.1</td>
</tr>
<tr>
<td>Σ Fatty acids</td>
<td>619.9</td>
<td>606.5-633.3</td>
<td>535.0-840.0</td>
</tr>
<tr>
<td><strong>Ratio’s</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22:5ω-6/C22:4ω-6</td>
<td>0.2</td>
<td>0.1-0.2</td>
<td>0.05-0.6</td>
</tr>
<tr>
<td>C22:5ω-6/C22:6ω-3</td>
<td>0.1</td>
<td>0.1-0.2</td>
<td>0.04-0.4</td>
</tr>
</tbody>
</table>

Figures represent means with 95% confidence intervals (CI). In case of skewed distribution geometric means were obtained. RV means laboratory reference values; Σ is summation; and Nd is non-detectable.

ᵃ Represent levels (+CI) exceeding the laboratory reference values.
3.2.2. Plasma

We found the following plasma FAs concentrations being below the lower limit of the normal reference range (see Table 3):

- In the ω-6 series: C22:4ω-6
- In the ω-9 series: C24:1ω-9 and C20:3ω-9

We found the following plasma FAs concentrations exceeding the upper limit of the normal reference range:

- In the ω-3 series: C18:3ω-3
- In the ω-6 series: C18:2ω-6 and C22:2ω-6
- In the ω-9 series: C18:1ω-9

In the saturated series: C14:0, C20:0 and C24:0

The sum of the FAs was above the upper limit of the normal range

The ratios C24:0/C22:0, C20:3ω-9/C20:4ω-6, C22:5ω-6/C22:4ω-6 and C22:5ω/C22:6ω-3 were all within normal limits.

Table 3: Plasma fatty acid concentrations (μmol)

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Mean</th>
<th>CI 95%</th>
<th>RV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ω-3 Series</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linolenic acid (18:3ω-3)</td>
<td>81.2a</td>
<td>70.2-94.0</td>
<td>30.0-70.0</td>
</tr>
<tr>
<td>Octadecatetraenoic acid (18:4ω-3)</td>
<td>Nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eicosapentaenoic acid (20:5ω-3)</td>
<td>68.3</td>
<td>57.6-79.0</td>
<td>15.0-95.0</td>
</tr>
<tr>
<td>Docosapentaenoic acid (22:5ω-3)</td>
<td>24.8</td>
<td>22.1-27.9</td>
<td>20.0-50.0</td>
</tr>
<tr>
<td>Docosahexaenoic acid (22:6ω-3)</td>
<td>127.7</td>
<td>115.4-140.1</td>
<td>75.0-180.0</td>
</tr>
<tr>
<td><strong>ω-6 Series</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (18:2ω-6)</td>
<td>4156.6a</td>
<td>3896.0-4417.2</td>
<td>1950.0-3500.0</td>
</tr>
<tr>
<td>Gamma-Linolenic acid (18:3ω-6)</td>
<td>47.8</td>
<td>39.1-58.5</td>
<td>15.0-50.0</td>
</tr>
<tr>
<td>Eicosadienoic acid (20:2ω-6)</td>
<td>20.7</td>
<td>18.6-22.7</td>
<td>10.0-40.0</td>
</tr>
<tr>
<td>Homogamma-Linolenic acid (20:3ω-6)</td>
<td>145.0</td>
<td>130.2-161.5</td>
<td>70.0-190.0</td>
</tr>
<tr>
<td>Arachidonic acid (20:4ω-6)</td>
<td>567.4</td>
<td>522.0-612.7</td>
<td>300.0-650.0</td>
</tr>
<tr>
<td>Docosadienoic acid (22:2ω-6)</td>
<td>13.9a</td>
<td>12.8-15.0</td>
<td>0.0-5.0</td>
</tr>
</tbody>
</table>

*Table is continued on the next page*
### Table 1: Fatty Acid Composition

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean</th>
<th>95% CI</th>
<th>RV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ω-6 Series</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Docosatetraenoic acid (22:4ω-6)</td>
<td>9.6a</td>
<td>8.5-10.8</td>
<td>10.0-25.0</td>
</tr>
<tr>
<td>Docosapentaenoic acid (22:5ω-6)</td>
<td>6.5</td>
<td>5.7-7.5</td>
<td>5.0-20.0</td>
</tr>
<tr>
<td><strong>ω-9 Series</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypogeic acid (16:1ω-9)</td>
<td>58.9</td>
<td>52.8-65.6</td>
<td>15.0-90.0</td>
</tr>
<tr>
<td>Oleic acid (18:1ω-9)</td>
<td>2607.9a</td>
<td>2342.8-2902.7</td>
<td>1035.0-2025.0</td>
</tr>
<tr>
<td>Gondoic acid (20:1ω-9)</td>
<td>12.5</td>
<td>11.0-14.2</td>
<td>10.0-25.0</td>
</tr>
<tr>
<td>Erucic acid (22:1ω-9)</td>
<td>Nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nervonic acid (24:1ω-9)</td>
<td>49.3a</td>
<td>46.5-52.1</td>
<td>55.0-85.0</td>
</tr>
<tr>
<td>Eicosatrienoic acid (20:3ω-9)</td>
<td>8.1a</td>
<td>6.9-9.5</td>
<td>10.0-20.0</td>
</tr>
<tr>
<td><strong>Saturates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristic acid (C14:0)</td>
<td>180.6a</td>
<td>148.1-220.2</td>
<td>50.0-145.0</td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>3015.1</td>
<td>2735.6-3323.0</td>
<td>1465.0-2790.0</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>712.7</td>
<td>651.6-779.5</td>
<td>465.0-755.0</td>
</tr>
<tr>
<td>Arachidic acid (C20:0)</td>
<td>33.4a</td>
<td>30.6-36.2</td>
<td>15.0-30.0</td>
</tr>
<tr>
<td>Behenic acid (C22:0)</td>
<td>43.0</td>
<td>40.6-45.4</td>
<td>40.0-100.0</td>
</tr>
<tr>
<td>Lignoceric acid (C24:0)</td>
<td>35.2</td>
<td>33.2-37.8</td>
<td>35.0-75.0</td>
</tr>
<tr>
<td><strong>Σ Fatty acids</strong></td>
<td>12718.3a</td>
<td>11718.2-13805.2</td>
<td>5950.0-11600.0</td>
</tr>
</tbody>
</table>

### Ratio's

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Mean</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C24:0/C22:0</td>
<td>0.8</td>
<td>0.8-0.9</td>
</tr>
<tr>
<td>C20:3ω-9/C20:4ω-6</td>
<td>0.02</td>
<td>0.01-0.02</td>
</tr>
<tr>
<td>C22:5ω-6/C22:4ω-6</td>
<td>0.7</td>
<td>0.6-0.8</td>
</tr>
<tr>
<td>C22:5ω-6/C22:6ω-3</td>
<td>0.06</td>
<td>0.05-0.07</td>
</tr>
</tbody>
</table>

Figures represent means with 95% confidence intervals (CI). In case of skewed distribution geometric means were obtained. RV means laboratory reference values; Σ is summation; and Nd is non-detectable.

* Represents levels (+CI) exceeding the laboratory reference values.

### 3.3. Associations between fatty acids and homocysteine

We found no associations between levels of the selected individual FAs in erythrocytes and thHcy except for C22:0 (β=0.419; P=0.010). However, after correction for multiple testing this association was no longer statistically significant.

We also found no associations between the Σ FA and the Σ of the ω-3, ω-6, ω-9 and the saturated FAs and thHcy.

In plasma associations between the following FAs and thHcy were found: C18:2ω-6 (β=0.404, P=0.01), C16:0 (β=0.324, P=0.05), C18:0 (β=0.361, P=0.03) and C22:0...
(β=0.332, P=0.05). After correction for multiple testing these associations were no longer statistically significant.

An association, albeit less strong after correction for multiple testing, was found between the Σ FA and tHcy (β=0.351, P=0.07) and between the Σ saturated FA (β=0.347, P=0.07) and tHcy. A significant association was found between the Σ ω-6 and tHcy (β=0.424, P=0.03).

The results obtained from the linear regression analysis did not alter when adjustment was made for the use of antidepressants.

3.4. Associations between fatty acids and depression
No associations between the depression status and the selected FAs in erythrocytes and plasma were found.

The strongest estimate for an association with the depression status was found for C18:1ω-9 levels above 82.4 pmol/10 e6 and C24:1ω-9 below 9.4 pmol/10 e6 (OR=9.8 (95% CI 1.089-88.229), P=0.042) in the erythrocytes, however statistical significance disappeared after correction was made for multiple testing.

4. Discussion
In this preliminary descriptive analysis of tHcy and fatty acids in patients with recurrent depression tHcy levels were within the laboratory reference values in both men and women. As was found in non-depressed individuals levels were lower in women than in men. This is in accordance with the normal vitamin B₆, B₁₂ and folic acid concentrations.

In the erythrocytes the following PUFAs were below the lower limit of the normal reference range: C22:5ω-3, C22:6ω-3, C22:4ω-6, C20:3ω-9 as was the MUFA C24:1ω-9. The PUFA C18:3ω-6 was slightly above the upper limit of the normal range. As the sum of the fatty acids was within the normal reference range, we do think that the alterations are best explained by decreased Δ-6 desaturase and elongase activities. The saturated fatty acids C20:0 and C22:0 were slightly below the lower limit of the reference range, probably reflecting the dietary intake and/or decreased elongase activity.

In plasma the following PUFAs were below the lower limit of the normal reference range: C20:3ω-9 and C24:1ω-9 again compatible with a Δ-6 desaturase and elongase defects, as was shown in the erythrocytes. The increases of C18:3ω-3, C18:2ω-6 as well as of C18:1ω-9,
the saturated FAs C14:0, C16:0, C20:0 and the Σ of the fatty acids most likely reflect dietary influences.

We did not find any association between plasma homocysteine and the Σ of the fatty acids, the Σ of the ω-3 and ω-9 PUFAs, the selected saturated FAs, the MUFAs or any of the members of the ω-3, ω-6 and ω-9 series in plasma or erythrocytes. The outcomes were not modulated by the use of antidepressants. The only significant relation we found was between the Σ of the ω-6 PUFAs in plasma and tHcy. This could be a reflection of the alleged interaction of an altered ω-6/ω-3 FA balance and tHcy 7.

In this preliminary study there was no association between the selected FAs in plasma or erythrocytes and the depressive status.

We do not think that the alterations we found in the members in the ω-3 and ω-9 series can be explained by dietary influences only. In other, controlled studies the decrease in DHA was not caused by dietary differences between depressive patients and control subjects 14, 16.

At least two other mechanisms may be involved: (1) a stress induced inhibition of Δ-5 and Δ-6 desaturase and elongase activities and (2) alterations in oxidative metabolism resulting in enhanced LPO. Both mechanisms, of course, may be enhanced by a dietary shift from ω-3 to ω-6 FAs.

Major depression is often accompanied by hypothalamic-pituitary-adrenal (HPA) axis dysfunction and elevated basal cortisol levels. The stress hormones ACTH, cortisol and adrenalin all depress Δ6 and Δ5 desaturases 30.

Oxidative stress arises whenever there is a disbalance in the mitochondrial generation of reactive oxygen species (ROS) and the intra- and extracellular antioxidative defense mechanisms 31. The carbon-atoms in a double bond are particularly sensitive to ROS, so the more double bonds the more susceptible the PUFA becomes to peroxidation 32. Stress is an important stimulus for ROS production. Also activation of immune cells by different causes induce an oxidative burst. Both depression and cardiovascular disease are accompanied by low grade inflammation. Omega-3 and ω-6 PUFAs and their respective eicosanoids (prostaglandins, thromboxanes and leukotrienes) have immunomodulatory properties. Omega-3 PUFAs and their derivatives have anti-inflammatory and ω-6 PUFAs pro-inflammatory actions. Thus a dietary shift from ω-3 to
\(\omega-6\) FAs will trigger enhanced activation of the immune system and an increased production of pro-inflammatory eicosanoids and cytokines \(^{33}\). Maes et al. reported significant lower vitamin E concentrations in patients with major depression suggesting lower antioxidant defenses against LPO \(^{34}\). In another study it was shown that (1) major depression, especially melancholia, was associated with elevated antioxidative enzyme activities (AEA) and LPO and (2) subchronic treatment with SSRIs had a suppressive effect on AEA and LPO \(^{35}\).

Very interestingly, the alterations we measured in the erythrocyte PUFAs in our patients with recurrent depression corresponded to those we reported previously in the erythrocyte membranes of young schizophrenic patients i.e. a decrease in C22:5\(\omega-3\), C22:6\(\omega-3\) and in C24:1\(\omega-9\) (nervonic acid) and C20:3\(\omega-9\) \(^{36}\). In addition reduced PUFAs (DHA and AA) and increased levels of LPO products were reported in never medicated, first-episode schizophrenic patients \(^{37}\).

Moreover, corresponding patterns of fatty acid changes are found in other unrelated diseases which have oxidative stress as a common feature e.g. diabetes, multiple sclerosis, Alzheimer’s disease and are also seen during normal aging \(^{38}, \; 39\). It could be hypothesized that oxidative stress induces an adaptive gene response. Pending on the level of oxidative stress, desaturase enzymes may “activated” or “inactivated” by products of PUFA peroxidation \(^{38}\).

DHA has a “paradoxical” role. Due to its high degree of unsaturation it is highly oxidizable, but experimental and clinical data support an antioxidative role for DHA. The lipid bound DHA in the bilayer does indeed trap ROS, but is able to limit further generation of LPO products \(^{40}\). However, DHA levels are critical; experimental and clinical data show that DHA may exhibit either pro-oxidant or antioxidant activities depending on the dosage of PUFA administered \(^{41}\).

Last but not least, the activities of the enzymes of the mitochondrial oxidative respiratory chain and level of oxidative stress are also genetically determined. Evidence for alterations in mitochondrial metabolism and by inference increased oxidative stress is growing steadily in schizophrenia and to a lesser extent in major depressive disorders \(^{42}, \; 43\).

At this stage we cannot draw firm conclusions as ours is an explorative descriptive study only. Major limitations are its cross-sectional design, and the fact that we could not adjust for important variables as the dietary composition, smoking and alcohol (ab)use. The study will be extended to the total cohort and be adjusted for important confounders (body mass, visceral fat, smoking, family history) as well as a comparison with a matched control group will be included. The usefulness of tHcy as an (in)direct parameter of oxidative metabolism needs to be evaluated further.
Nevertheless, we found differences in the fatty acids despite the clinical recovery of most of our patients, and the fact that 66% of them used antidepressants. In this, our data correspond with a previous study in which it was shown that FA alterations may persist despite successful antidepressant treatment. So, persistent FA changes may represent a biological “trait” marker for (relapse or recurrency of) depression.

Finally, in prospective studies FA metabolism should be studied together with parameters of oxidative metabolism for a better understanding and interpretation of alterations in fatty acid metabolism.

Acknowledgements
We would like to thank Dr. T.A. Egelte for his expert, critical and constructive comments.
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Time after Time; biological factors in the course of recurrent depression


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Fatty acid metabolism

4.2 Plasma and Erythrocyte Fatty Acid Patterns in Patients with Recurrent Depression: A Matched Case-Control Study

Assies J, Pouwer F, Lok A, Mocking RJ, Bockting CL, Visser I, Abeling NG, Duran M, Schene AH

Abstract

Background
The polyunsaturated fatty acid (PUFA) composition of (nerve) cell membranes may be involved in the pathophysiology of depression. Studies so far, focussed mainly on omega-3 and omega-6 PUFAs. In the present study, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and PUFAs of the omega-3, -6 and -9 series in plasma and erythrocytes of patients with recurrent major depressive disorder (MDD-R) were compared with controls.

Methodology and Principal Findings
We carried out a case-control study. The sample consisted of 137 patients with MDD-R and 65 matched non-depressed controls. In plasma and erythrocytes of patients with MDD-R the concentrations of most of the SFAs and MUFAs, and additionally erythrocyte PUFAs, all with a chain length >20 carbon (C) atoms, were significantly lower than in the controls. In contrast, the concentrations of most of the shorter chain members (≤18C) of the SFAs and MUFAs were significantly higher in the patients. Estimated activities of several elongases in plasma of patients were significantly altered, whereas delta-9 desaturase activity for C14:0 and C18:0 was significantly higher.

Conclusions/Significance
The fatty acid status of patients with MDD-R not only differs with regard to omega-3 and omega-6 PUFAs, but also concerns other fatty acids. These alterations may be due to: differences in diet, changes in synthesizing enzyme activities, higher levels of chronic (oxidative) stress but may also result from adaptive strategies by providing protection against enhanced oxidative stress and production of free radicals.
Introduction

Major depressive disorder (MDD) - in particular its recurrent, chronic form (MDD-R) - is ranked as a major cause of disability and excess mortality worldwide. Moreover, mortality studies indicate that cardiovascular disease (CVD) accounts for more excess death in MDD patients than any other single cause.

Fatty acids (FAs) may play a key role in the pathogenesis of both MDD and CVD and so could explain their mutual association. Reported alterations in FAs in MDD include a low omega (ω)-3 polyunsaturated fatty acid (PUFA) intake, a decrease in ω-3 PUFAs and increased ω-6/ω-3 PUFA ratios in plasma, erythrocytes, adipose tissue and post-mortem brain tissue. The nature of these FA alterations still has to be elucidated.

PUFAs are essential constituents of the human brain and involved in the regulation of cognition and emotion. PUFAs of the ω-3 and ω-6 series are key components of (nerve) cell membrane phospholipids (PLs) and synapses and are responsible for: signal transduction, ion transport and receptor sensitivity (e.g. for serotonin, dopamine, endocannabinoids). Important members of the ω-3 and ω-6 series are eicosapentaenoic acid (EPA, C20:5 ω-3) and arachidonic acid (AA, C20:4 ω-6) which are precursors for eicosanoids (prostaglandins, leukotrienes, thromboxanes) which mediate infection, inflammation and haemostasis. Importantly, AA derived eicosanoids have a stimulatory effect while EPA derived eicosanoids have a suppressive effect on these processes. Furthermore, docosahexaenoic acid (DHA, C22:6 ω-3) derived docosanoids (resolvins, neuroprotectins) have a neuroprotective effect.

Humans are dependent on their diet for the intake of the two major 18 carbon (C) chain precursors of the ω-3 and ω-6 series of FAs: α-linolenic acid (ALA, C18:3 ω-3) and linoleic acid (LA, C18:2 ω-6). Both ALA and LA are transformed by elongases and desaturases to longer chain PUFAs containing three to six double bonds (Fig. 1).

Delta (Δ)6 and Δ5 desaturases introduce double bonds in the ω-3 and ω-6 PUFAs; Δ9 desaturases synthesize monounsaturated fatty acids (MUFA) from saturated fatty acids (SFA). Elongation to long chain (LC) FAs with up to 22 Cs and very long chain (VLC) FAs with >22 Cs occurs in the endoplasmatic reticulum by elongases. Because there is only limited (<15%) conversion of ALA to EPA and DHA and from LA to AA, while the ω-3 and ω-6 PUFAs compete for the same desaturases and elongases, a well balanced ω-6/ω-3 diet is important to reach a sufficient FA status. An ω-6/ω-3 PUFA ratio of <4:1 is thought to represent a healthy
balance. Fatty fish is the major source of ω-3 PUFAs in humans. In Western industrialized coun-
tries increases in dietary ω-6 FA intake and reductions in ω-3 FA has changed the ω-6/ω-3 FA
ratio to an estimated 15:1 15.

Figure 1: Pathways of fatty acid metabolism

Due to figure dimension restrictions, the conversion of C16:0 to C16:1 ω-7 could not be depicted.

Abbreviations - Elo = Elongase; D = Desaturase.
Plasma FA levels reflect recent dietary intake while longer-term dietary impact is better reflected in erythrocyte (membrane) and adipose tissue FA composition. Moreover FA status depends not only on dietary intake, but also on endogenous metabolism. The relationship between intake and incorporation into peripheral tissues was found to be non-linear and modulated by genetic factors, age, gender and oxidative stress generated by life style (stress, smoking, use of alcohol, physical activity).

An important limitation of earlier studies on FA levels in MDD is that they mostly addressed only ω-3 and ω-6 PUFA levels. They did not measure the whole FA spectrum, neither were estimates of their respective desaturases and elongases routinely reported. Moreover, these studies focussed mainly on patients suffering from a single depressive episode while MDD is increasingly considered as a recurrent and often chronic disorder rather than a single episode disorder. Data regarding the FA metabolism of patients with recurrent depression is currently lacking. We hypothesize that compared to non-depressed controls- patients with MDD-R would have lower PUFA levels and a higher ω-6/ω-3 ratio and that these alterations are “trait” dependent, i.e. independent of the current depressive status.

We previously compared FA levels in plasma and erythrocytes of 44 patients randomly chosen out of a cohort of 137 MDD-R patients with laboratory reference values. We subsequently analyzed the FA spectrum and estimated activities of their respective desaturases and elongases of the whole cohort and compared them with a matched healthy control group (n = 65).

Materials and Methods
Population
Recurrently depressed patients participated in a randomized clinical trial comparing the efficacy of preventive cognitive therapy on relapse and recurrence (DELTA Study). The background and methodology have been described elsewhere. In brief, the inclusion criteria of the trial were: age between 18 and 65 years, at least two depressive episodes in the previous 5 years, and having reached current remission status according to the criteria of fourth edition of the Diagnostic and Statistical Manual. Exclusion criteria were current or previous mania or hypomania (bipolar illness), any psychotic disorder (current or previous), organic brain damage, alcohol or drug abuse, predominant anxiety disorder, recent ECT, recent cognitive treatment or receiving CT at the start of the study or current psychotherapy with a frequency of more than two times a month. The medical ethical committee of the Academic Medical Centre approved the study protocol.
At the 2-years follow up of the original trial, the patients participated in the current study, so patients were either in a relapse (depressed) or a remission (non-depressed). Furthermore, we recruited age and gender matched, healthy, non-depressed control subjects by advertising. All participants provided written informed consent prior to enrolment.

Assessment of depression and other variables
For the current study the psychiatric status was assessed with the Structured Clinical Interview of DSM-IV disorders (SCID-I) by trained interviewers 19. Information about the use of antidepressants during sampling was obtained by the Trimbos/IMTA questionnaire for Costs associated with psychiatric illness (TIC-P:40) and by interview 20. Furthermore, blood was sampled in the non-fasting state and anthropometric measurements were taken, including; body mass index (BMI; kg/m2), WC in cm and waist to hip ratio (WHR). WC was measured at the level midway between lower rib and the iliac crest, with participants in standing position.

Blood sample collection and analysis of FAs
FAs in plasma and washed erythrocytes were analyzed by capillary gas chromatography, as described previously 18, 21. Plasma was separated within 4 h of collection and stored at −80°C until analysis. Total FAs in plasma and washed erythrocytes were expressed as µmol/L and pmol/10e6 erythrocytes respectively. FAs were analyzed both quantitatively and qualitatively. Qualitative analysis of FAs as a percentage of total FAs may be misleading; because the consequence of this approach is that an increase in one FA results in a relative decrease in other FAs to maintain 100% 16. Therefore we chose to present the results in concentrations. The analysis in percentages is given in supplementary tables (S1, S2). The estimated activities (or surrogate measures) of Δ5, Δ6 and Δ9 desaturases and elongases in plasma are expressed as product/precursor ratios. We do not estimate these activities in erythrocytes because they are reported to be incapable of chain elongation or desaturation of FAs 16.
Table S1: Plasma fatty acid concentrations (% of total fatty acids) of MDD-R patients compared with a matched non-depressed control groupa

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Controls (n = 65)</th>
<th>MDD-R (n = 137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic acid (C18:2 ω-6)</td>
<td>29.3 ± 4.3</td>
<td>30.6 ± 5.0</td>
</tr>
<tr>
<td>Gamma-linolenic acid (C18:3 ω-6)</td>
<td>0.5 ± 0.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Homogamma linolenic acid (C20:3 ω-6)</td>
<td>1.5 ± 0.7</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4 ω-6)</td>
<td>5.3 ± 1.3</td>
<td>4.8 ± 1.2</td>
</tr>
<tr>
<td>Docosatetraenoic acid (C22:4 ω-6)</td>
<td>0.13 ± 0.04</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>Docosapentaenoic acid (C22:5 ω-6)</td>
<td>0.09 ± 0.03</td>
<td>0.07 ± 0.04**</td>
</tr>
<tr>
<td>Eicosadienoic acid (C20:2 ω-6)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Docosadienoic acid (C22:2 ω-6)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Myristoleic acid (C14:1 ω-5)</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2***</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1 ω-7)</td>
<td>2.4 ± 1.0</td>
<td>2.9 ± 1.3***</td>
</tr>
<tr>
<td>Vaccenic acid (C18:1 ω-7)</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.3</td>
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<tr>
<td>13-eicosenoic acid (C20:1 ω-7)</td>
<td>0.12 ± 0.05</td>
<td>0.07 ± 0.13***</td>
</tr>
<tr>
<td>Hypogeic acid (C16:1 ω-9)</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1**</td>
</tr>
<tr>
<td>Oleic acid (C18:1 ω-9)</td>
<td>18.8 ± 3.2</td>
<td>19.3 ± 3.0</td>
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<tr>
<td>Gondoic acid (C20:1 ω-9)</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Erucic acid (C22:1 ω-9)</td>
<td>0.11 ± 0.08</td>
<td>0.04 ± 0.08***</td>
</tr>
<tr>
<td>Nervonic acid (C24:1 ω-9)</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.2***</td>
</tr>
<tr>
<td>Eicosatrienoic acid (C20:3 ω-9)</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Myristic acid (C14:0)</td>
<td>1.4 ± 0.5</td>
<td>1.6 ± 0.7**</td>
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<tr>
<td>Pentadecanoic acid (C15:0)</td>
<td>0.29 ± 0.06</td>
<td>0.15 ± 0.16***</td>
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<td>Palmitic acid (C16:0)</td>
<td>25.6 ± 1.8</td>
<td>25.3 ± 3.3</td>
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<tr>
<td>Stearic acid (C18:0)</td>
<td>7.2 ± 0.7</td>
<td>6.4 ± 1.1***</td>
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<td>Arachidic acid (C20:0)</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
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<td>Behenic acid (C22:0)</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1***</td>
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<tr>
<td>Lignoceric acid (C24:0)</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1***</td>
</tr>
</tbody>
</table>

a Independent means t-tests: significantly different in comparison to controls at * p<.05, ** p<.01, *** p<.001.
Table S2: Erythrocyte fatty acid percentages (% of total fatty acids) of MDD-R patients compared with a matched non-depressed control group

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Controls (n = 65)</th>
<th>MDD-R (n = 137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linolenic acid (C18:3 ω-3)</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Octadecatetraenoic acid (C18:4 ω-3)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (C20:5 ω-3)</td>
<td>0.6 ± 0.3</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Docosapentaenoic acid (C22:5 ω-3)</td>
<td>1.7 ± 0.3</td>
<td>1.4 ± 0.3 ***</td>
</tr>
<tr>
<td>Docosahexaenoic acid (C22:6 ω-3)</td>
<td>3.2 ± 1.0</td>
<td>2.5 ± 0.8 **</td>
</tr>
<tr>
<td>Linoleic acid (C18:2 ω-6)</td>
<td>10.8 ± 1.3</td>
<td>11.3 ± 1.6</td>
</tr>
<tr>
<td>Gamma-linolenic acid (C18:3 ω-6)</td>
<td>0.1 ± 0.06</td>
<td>0.1 ± 0.04</td>
</tr>
<tr>
<td>Homogamma linolenic acid (C20:3 ω-6)</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.4 *</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4 ω-6)</td>
<td>13.2 ± 1.0</td>
<td>12.2 ± 1.6 ***</td>
</tr>
<tr>
<td>Docosatetraenoic acid (C22:4 ω-6)</td>
<td>1.8 ± 0.5</td>
<td>0.3 ± 0.1 ***</td>
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<td>Docosadienoic acid (C22:2 ω-6)</td>
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<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Myristoleic acid (C14:1 ω-5)</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1 ***</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1 ω-7)</td>
<td>0.4 ± 0.2</td>
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</tr>
<tr>
<td>Vaccenic acid (C18:1 ω-7)</td>
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</tr>
<tr>
<td>13-eicosenoic acid (C20:1 ω-7)</td>
<td>0.04 ± 0.06</td>
<td>0.03 ± 0.05 ***</td>
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<td>Hypogeic acid (C16:1 ω-9)</td>
<td>0.3 ± 0.5</td>
<td>0.2 ± 0.1 *</td>
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<td>0.3 ± 0.4 ***</td>
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<td>Myristic acid (C14:0)</td>
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</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>16.9 ± 0.8</td>
<td>17.6 ± 0.8</td>
</tr>
<tr>
<td>Arachidic acid (C20:0)</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1 ***</td>
</tr>
<tr>
<td>Behenic acid (C22:0)</td>
<td>1.5 ± 0.2</td>
<td>1.3 ± 0.3 ***</td>
</tr>
<tr>
<td>Lignoceric acid (C24:0)</td>
<td>3.4 ± 0.4</td>
<td>2.6 ± 0.8 **</td>
</tr>
<tr>
<td>Lignoceric acid (C24:0)</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1 ***</td>
</tr>
</tbody>
</table>

a Independent means t-tests: significantly different in comparison to controls at * p<.05, ** p<.01, *** p<.001.
Statistical analysis

Statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Continuous demographic data of the patients with MDD-R and non-depressed matched controls were analyzed using independent means t-tests and \( \chi^2 \) tests were used for categorical variables. Additional tests with correction for WC were performed by the use of ANCOVA’s. ANOVA’s were also performed in additional, explorative analyses to test whether FA concentrations differed between patients with continuous, intermittent or without use of anti-depressant medication. Independent means t-tests were performed to detect differences in FA concentrations between currently depressed and non-depressed patients.

In FA research, multiple tests have to be performed because of the many members of the different FA series. Therefore the results should be interpreted cautiously with regard to type I errors. We have chosen not to adjust our p-value for multiple testing for the following reasons. First, although our research was partly exploratory (with regard to FA series other than the \( \omega-3 \) and \( \omega-6 \)), the main part of the analyses (the \( \omega-3 \) and \( \omega-6 \) series) was hypothesis driven. Second, it is not common practice in FA research to adjust the p-value for multiple testing. Third, adjustment for multiple testing may induce type II errors.

Results

Sample characteristics of the patients and the non-depressed control group are displayed in Table 1. Body weight, BMI, WC and WHR were significantly higher in MDD-R patients, compared to non-depressed controls. Patients had a lower education level, smoking habits did not differ. Information on anti-depressant use of the MDD-R patients is given in Table 2.

<table>
<thead>
<tr>
<th>Table 1: Demographic and clinical characteristics of MDD-R patients and the matched non-depressed control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 65)</td>
</tr>
<tr>
<td>Age (years ± SD)</td>
</tr>
<tr>
<td>Male sex (% (n/N))</td>
</tr>
<tr>
<td>Weight (kg ± SD)</td>
</tr>
<tr>
<td>BMI ± SD</td>
</tr>
<tr>
<td>WC (cm ± SD)</td>
</tr>
<tr>
<td>WHR ± SD</td>
</tr>
<tr>
<td>Smoking (% (n/N))</td>
</tr>
</tbody>
</table>

*Table is continued on the next page*
Time after Time; biological factors in the course of recurrent depression

<table>
<thead>
<tr>
<th>Education (% (high/middle/low))</th>
<th>74.6/20.3/5.1</th>
<th>35.3/30.9/33.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current depression (%)</td>
<td>0% (0/65)</td>
<td>19% (26/136)</td>
</tr>
</tbody>
</table>

Abbreviations - MDD-R = Major Depressive Disorder, Recurrent form; BMI = Body Mass Index; WC = Waist Circumference; WHR = Waist-to-Hip Ratio.

Independent means t-tests or χ² tests: significantly different in comparison to controls at * p<.05, ** p<.01, *** p<.001.

Table 2: Anti-depressant use of MDD-R patients

<table>
<thead>
<tr>
<th>Subcategory</th>
<th>Result (% (n/N))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current use of anti-depressants</td>
<td>63% (81/129)</td>
</tr>
<tr>
<td>Type of current anti-depressant</td>
<td></td>
</tr>
<tr>
<td>TCA</td>
<td>9% (7/81)</td>
</tr>
<tr>
<td>SSRI</td>
<td>65% (53/81)</td>
</tr>
<tr>
<td>SNRI</td>
<td>19% (15/81)</td>
</tr>
<tr>
<td>Antidepressant and/or lithium</td>
<td>6% (5/81)</td>
</tr>
<tr>
<td>Other</td>
<td>1% (1/81)</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>22.6% (14/62)</td>
</tr>
<tr>
<td>Education (% (high/middle/low))</td>
<td>74.6/20.3/5.1</td>
</tr>
<tr>
<td>Current depression (%)</td>
<td>0% (0/65)</td>
</tr>
</tbody>
</table>

Abbreviations - TCA = Tricyclic Anti-Depressants; SSRI = Selective Serotonin Reuptake Inhibitors; SNRI = Serotonin Noradrenaline Reuptake Inhibitors.

FA concentrations in plasma (Table 3)
The sums of total FAs, SFAs, MUFAs and PUFAs were all significantly (p<.001) higher in the recurrently depressed patients than in the non-depressed control subjects.

The sum of the FAs of the ω-3 series in plasma did not differ between patients and controls; the concentration of the precursor C18:3 ω-3, was higher (p<.05), while levels of EPA and DHA were similar in patients and controls. The sum of ω-6 PUFAs was significantly higher in patients (p<.001) and the concentration of the ω-6 series precursor linoleic acid (C18:2 ω-6) and the next member (C18:3 ω-6) were significantly higher in the patients (p<.001, p<.05). The concentrations of other members of the ω-6 series did not differ significantly between patients and controls.

The concentration of the MUFA C14:1 ω-5 was significantly higher in patients’ plasma than in controls (p<.001). The sum of ω-7 MUFAs was significantly higher in the patients (p<.001)
as were the first two members of the ω-7 series, C16:1 ω-7 and C18:1 ω-7, but C20:1 ω-7 was similar in patients and controls. The sum of ω-9 MUFAs and the first two members C16:1 ω-9 and C18:1 ω-9 were all significantly higher in the patients than in controls (p<.001). The concentration of C20:1 ω-9 was similar, but levels of C22:1 ω-9 and C24:1 ω-9 were both significantly lower in the patients (p<.001).

The plasma SFAs concentrations of C14:0 and C18:0 were both significantly higher (p<.001), but concentrations of C20:0 and C22:0 were significantly lower in patients than in controls (p<.01).

Table 3: Plasma fatty acid concentrations (µmol/l) of MDD-R patients compared with a matched non-depressed control group

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Controls (n = 65)</th>
<th>MDD-R (n = 137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linolenic acid (C18:3 ω-3)</td>
<td>63±40</td>
<td>79±45*</td>
</tr>
<tr>
<td>Octadectetraenoic acid (C18:4 ω-3)</td>
<td>1.5±1.9</td>
<td>1.4±2.6</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (C20:5 ω-3)</td>
<td>67±44</td>
<td>73±44</td>
</tr>
<tr>
<td>Docosapentaenoic acid (C22:5 ω-3)</td>
<td>34±11</td>
<td>31±12</td>
</tr>
<tr>
<td>Docosahexaenoic acid (C22:6 ω-3)</td>
<td>130±53</td>
<td>125±47</td>
</tr>
<tr>
<td>Σ omega-3 PUFAs</td>
<td>295±110</td>
<td>309±111</td>
</tr>
<tr>
<td>Linoleic acid (C18:2 ω-6)</td>
<td>3040±685</td>
<td>3629±894***</td>
</tr>
<tr>
<td>Gamma-linolenic acid (C18:3 ω-6)</td>
<td>47±20</td>
<td>57±34*</td>
</tr>
<tr>
<td>Homogamma linolenic acid (C20:3 ω-6)</td>
<td>156±94</td>
<td>159±65</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4 ω-6)</td>
<td>554±165</td>
<td>572±162</td>
</tr>
<tr>
<td>Docosatetraenoic acid (C22:4 ω-6)</td>
<td>14±5</td>
<td>14±6</td>
</tr>
<tr>
<td>Docosapentaenoic acid (C22:5 ω-6)</td>
<td>9±4</td>
<td>9±5</td>
</tr>
<tr>
<td>Eicosadienoic acid (C20:2 ω-6)</td>
<td>19±10</td>
<td>21±7</td>
</tr>
<tr>
<td>Docosadienoic acid (C22:2 ω-6)</td>
<td>0.0±0.0</td>
<td>5.2±7.2***</td>
</tr>
<tr>
<td>Σ omega-6 PUFAs</td>
<td>3840±785</td>
<td>4481±1015***</td>
</tr>
<tr>
<td>Myristoleic acid (C14:1 ω-5)</td>
<td>8.8±7.5</td>
<td>21.3±23.5***</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1 ω-7)</td>
<td>242±124</td>
<td>388±280**</td>
</tr>
<tr>
<td>Vaccenic acid (C18:1 ω-7)</td>
<td>163±46</td>
<td>198±63***</td>
</tr>
<tr>
<td>13-eicosenoic acid (C20:1 ω-7)</td>
<td>13±7</td>
<td>9±21</td>
</tr>
<tr>
<td>Σ omega-7 PUFAs</td>
<td>418±168</td>
<td>595±333***</td>
</tr>
<tr>
<td>Hypogeic acid (C16:1 ω-9)</td>
<td>42±15</td>
<td>55±23***</td>
</tr>
<tr>
<td>Oleic acid (C18:1 ω-9)</td>
<td>1975±722</td>
<td>2439±1033***</td>
</tr>
</tbody>
</table>

Table is continued on the next page >
### FAs in erythrocytes (Table 4)

Compared to healthy controls, the sums of total FAs and PUFAs were significantly (p<.001) lower in the MDD-R patients, while the sums of SFAs and MUFAs were similar.

The sum of ω-3 PUFAs in erythrocytes was significantly lower in patients than in controls (p<.001). The ω-3 precursor C18:3 ω-3 did not differ but its Δ6 desaturase product C18:4 ω-3 was significantly higher in patients than in controls (p<.001). EPA concentrations were not different but C22:5 ω-3 -the elongase product of EPA- and DHA were both significantly lower in the patients (p<.001).

Analogous to the sum of ω-3 PUFAs, the sum of ω-6 PUFAs in the erythrocytes was also significantly lower in the patients than in the control subjects (p<.001). The concentration of the precursor C18:2 ω-6 was similar, while the concentration of its Δ6 desaturase product C18:3 ω-6 was significantly higher in patients than in controls (p<.001). The subsequent members of the ω-6 series: AA, C22:4 ω-6 and C22:5 ω-6 were all significantly lower (p<.001) in the patients.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Mean ± SD Controls</th>
<th>Mean ± SD Patients</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gondoic acid (C20:1 ω-9)</td>
<td>14±6</td>
<td>15±8</td>
<td></td>
</tr>
<tr>
<td>Erucid acid (C22:1 ω-9)</td>
<td>12±8</td>
<td>4±7***</td>
<td></td>
</tr>
<tr>
<td>Nervonic acid (C24:1 ω-9)</td>
<td>64±20</td>
<td>54±12***</td>
<td></td>
</tr>
<tr>
<td>Eicosatrienoic acid (C20:3 ω-9)</td>
<td>10±5</td>
<td>10±7</td>
<td></td>
</tr>
<tr>
<td>Σ omega-9 PUFAs</td>
<td>2110±754</td>
<td>2578±1057***</td>
<td></td>
</tr>
<tr>
<td>Myristic acid (C14:0)</td>
<td>146±74</td>
<td>213±163***</td>
<td></td>
</tr>
<tr>
<td>Pentadecanoic acid (C15:0)</td>
<td>30±9</td>
<td>34±16</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>2651±707</td>
<td>3163±1271***</td>
<td></td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>741±174</td>
<td>787±242</td>
<td></td>
</tr>
<tr>
<td>Arachidic acid (C20:0)</td>
<td>25.5±6.1</td>
<td>28.5±7.9”</td>
<td></td>
</tr>
<tr>
<td>Behenic acid (C22:0)</td>
<td>58±12</td>
<td>53±13”</td>
<td></td>
</tr>
<tr>
<td>Lignoceric acid (C24:0)</td>
<td>38±8</td>
<td>37±9</td>
<td></td>
</tr>
<tr>
<td>Σ Saturated fatty acids</td>
<td>3660±942</td>
<td>4297±1642***</td>
<td></td>
</tr>
<tr>
<td>Σ Monounsaturated fatty acids</td>
<td>2527±881</td>
<td>3183±1365***</td>
<td></td>
</tr>
<tr>
<td>Σ Polyunsaturated fatty acids</td>
<td>4145±837</td>
<td>4801±1077***</td>
<td></td>
</tr>
<tr>
<td>Σ Total fatty acids</td>
<td>10382±2442</td>
<td>12273±3836***</td>
<td></td>
</tr>
</tbody>
</table>

* Independent means t-tests: significantly different in comparison to controls at ’ p<.05, ** p<.01, *** p<.001.

b C15:0 excluded due to missing values.
The concentration of the erythrocyte MUFA C14:1 ω-5 was significantly lower in the patients than in the control subjects (p<.001). In the ω-9 series C24:1 ω-9 (nervonic acid) and the sum of the ω-9 FAs were significantly lower in the patients (p<.001).

The levels of SFAs C14:0 and C18:0 were similar but levels of C20:0, C22:0 and C24:0 were all significantly lower in the patients (p<.001).

Table 4: Erythrocyte fatty acid concentrations (pmol/10e6 erythrocytes) of MDD-R patients compared with a matched non-depressed control group

<table>
<thead>
<tr>
<th>Fatty Acid Description</th>
<th>Controls (n = 65)</th>
<th>MDD-R (n = 137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linolenic acid (C18:3 ω-3)</td>
<td>0.81±0.32</td>
<td>0.84±0.31</td>
</tr>
<tr>
<td>Octadectetraenoic acid (C18:4 ω-3)</td>
<td>0.035±0.11</td>
<td>0.21±0.27”***</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (C20:5 ω-3)</td>
<td>3.9±2.0</td>
<td>3.3±1.6</td>
</tr>
<tr>
<td>Docosapentaenoic acid (C22:5 ω-3)</td>
<td>10.5±2.1</td>
<td>7.9±1.5”***</td>
</tr>
<tr>
<td>Docosahexaenoic acid (C22:6 ω-3)</td>
<td>20.1±6.6</td>
<td>14.8±4.2”***</td>
</tr>
<tr>
<td>Σ omega-3 PUFAs</td>
<td>35.3±10</td>
<td>26.9±6.4”***</td>
</tr>
<tr>
<td>Linoleic acid (C18:2 ω-6)</td>
<td>67±12</td>
<td>66±13</td>
</tr>
<tr>
<td>Gamma-linolenic acid (C18:3 ω-6)</td>
<td>0.38±0.37</td>
<td>0.57±0.21”***</td>
</tr>
<tr>
<td>Homogamma linolenic acid (C20:3 ω-6)</td>
<td>9.7±2.1</td>
<td>8.8±2.4*</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4 ω-6)</td>
<td>81.6±8.7</td>
<td>71.5±10.5”***</td>
</tr>
<tr>
<td>Docosatetraenoic acid (C22:4 ω-6)</td>
<td>13.0±2.5</td>
<td>10.7±2.6”**</td>
</tr>
<tr>
<td>Docosapentaenoic acid (C22:5 ω-6)</td>
<td>2.1±0.6</td>
<td>1.7±0.6”***</td>
</tr>
<tr>
<td>Eicosadienoic acid (C20:2 ω-6)</td>
<td>1.3±0.4</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>Docosadienoic acid (C22:2 ω-6)</td>
<td>0±0</td>
<td>0.37±0.43”***</td>
</tr>
<tr>
<td>Σ omega-6 PUFAs</td>
<td>175±19.9</td>
<td>161.4±19.0”**</td>
</tr>
<tr>
<td>Myristoleic acid (C14:1 ω-5)</td>
<td>0.60±0.66</td>
<td>0.25±0.31”***</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1 ω-7)</td>
<td>2.5±1.3</td>
<td>3.0±1.5’</td>
</tr>
<tr>
<td>Vaccenic acid (C18:1 ω-7)</td>
<td>7.9±1.4</td>
<td>7.5±1.5</td>
</tr>
<tr>
<td>13-eicosenoic acid (C20:1 ω-7)</td>
<td>0.27±0.35</td>
<td>0.21±0.29</td>
</tr>
<tr>
<td>Σ omega-7 PUFAs</td>
<td>10.6±2.3</td>
<td>10.6±2.5</td>
</tr>
<tr>
<td>Hypogeanic acid (C16:1 ω-9)</td>
<td>1.9±3.0</td>
<td>0.9±0.6’</td>
</tr>
<tr>
<td>Oleic acid (C18:1 ω-9)</td>
<td>75±10</td>
<td>74±10</td>
</tr>
<tr>
<td>Gondoic acid (C20:1 ω-9)</td>
<td>1.2±0.4</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>Erucid acid (C22:1 ω-9)</td>
<td>2.1±1.4</td>
<td>1.9±2.2</td>
</tr>
<tr>
<td>Nervonic acid (C24:1 ω-9)</td>
<td>19.6±3.5</td>
<td>13.3±3.4”***</td>
</tr>
</tbody>
</table>

Table is continued on the next page >
Eicosatrienoic acid (C20:3 ω-9)  0.31±0.24  0.36±0.24
Σ omega-9 PUFAs  100±13  92±10***
Myristic acid (C14:0)  3.5±1.2  3.3±1.0
Palmitic acid (C16:0)  156±19  164±23*
Stearic acid (C18:0)  104±11  103±11
Arachidic acid (C20:0)  2.8±0.5  2.5±0.4***
Behenic acid (C22:0)  9.5±1.6  7.6±1.5***
Lignoceric acid (C24:0)  21.3±3.0  14.8±4.2***
Σ Saturated fatty acids  297±32  295±29
Σ Monounsaturated fatty acids  112±14  112±14
Σ Polyunsaturated fatty acids  211±23  189±18***
Σ Total fatty acids  620±66  588±54***
Σ Total fatty acids  10382±2442  12273±3836***

* Independent means t-tests: significantly different in comparison to controls at * p<.05, ** p<.01, *** p<.001.

Ratios, desaturases and elongases (Table 5, 6, 7)
The various ratios between the sums of SFA, MUFAs, PUFAs, ω-3 and ω-6 PUFAs and AA/EPA and AA/DHA in the plasma did not differ between the patients and control subjects (Table 5). In the erythrocyte, the ratios of the Σ SFA/Σ PUFA, the Σ MUFA/Σ PUFA, and Σ SFA/Σ MUFA and the Σ ω-6/Σ ω-3 PUFAs were higher in the patients but the AA/EPA and the AA/DHA ratios did not differ (Table 6).

Table 5: Several ratios of fatty acids concentrations in plasma of MDD-R patients compared with a matched non-depressed control group

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 65)</th>
<th>MDD-R (n = 137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Σ SFAs/Σ PUFAs</td>
<td>1.154±0.169</td>
<td>1.172±0.232</td>
</tr>
<tr>
<td>Σ MUFAs/Σ PUFAs</td>
<td>0.603±0.147</td>
<td>0.652±0.178</td>
</tr>
<tr>
<td>Σ SFAs/Σ MUFAs</td>
<td>0.885±0.143</td>
<td>0.889±0.202</td>
</tr>
<tr>
<td>Σ omega-6/Σ omega-3</td>
<td>14.37±4.59</td>
<td>15.79±4.80</td>
</tr>
<tr>
<td>C20:4 ω-6/C20:5 ω-3</td>
<td>11.56±7.38</td>
<td>10.58±6.20</td>
</tr>
<tr>
<td>C20:4 ω-6/C22:6 ω-3</td>
<td>4.887±2.366</td>
<td>5.010±1.7062</td>
</tr>
</tbody>
</table>

* Independent means t-tests: significantly different in comparison to controls at * p<.05, ** p<.01, *** p<.001.
Table 6: Several ratios of fatty acids concentrations in erythrocytes of MDD-R patients compared with a matched non-depressed control group

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 65)</th>
<th>MDD-R (n = 137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Σ SFAs/Σ PUFAs</td>
<td>1.414±0.065</td>
<td>1.572±0.128***</td>
</tr>
<tr>
<td>Σ MUFAs/Σ PUFAs</td>
<td>0.529±0.043</td>
<td>0.548±0.057**</td>
</tr>
<tr>
<td>Σ SFAs/Σ MUFAs</td>
<td>2.684±1.773</td>
<td>2.884±0.232***</td>
</tr>
<tr>
<td>Σ omega-6/Σ omega-3</td>
<td>5.437±2.363</td>
<td>6.377±1.773***</td>
</tr>
<tr>
<td>C20:4 ω-6/C20:5 ω-3</td>
<td>27.45±16.79</td>
<td>26.41±12.71</td>
</tr>
<tr>
<td>C20:4 ω-6/C22:6 ω-3</td>
<td>5.149±5.863</td>
<td>5.232±1.701</td>
</tr>
</tbody>
</table>

* Independent means t-tests: significantly different in comparison to controls at * p<.05, **p<.01, ***p<.001.

The estimated activities of desaturases and elongases in plasma are given in Table 7. Δ6 and Δ5 desaturases did not differ in their activity. The estimated activity of the Δ9 desaturases (C14:1 ω-5/C14:0 and C18:1 ω-9/C18:0) was significantly higher in patients than in the control subjects (p<.001), but C24:1 ω-9/C24:0 was significantly lower (p<.01).

Table 7: Several indices of desaturases and elongases in plasma of MDD-R patients compared with a matched non-depressed control group

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 65)</th>
<th>MDD-R (n = 137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta-6 omega-3 (C18:4n-3/C18:3n-3)</td>
<td>0.029±0.039</td>
<td>0.023±0.039</td>
</tr>
<tr>
<td>Delta-5 omega-3 (C20:5n-3/C18:4n-3)</td>
<td>25.25±17.56</td>
<td>26.96±21.6</td>
</tr>
<tr>
<td>Delta-6 omega-3 (C22:6n-3/C22:5n-3)</td>
<td>3.99±1.44</td>
<td>4.33±1.49</td>
</tr>
<tr>
<td>Elongase (C22:5n-3/C20:5n-3)</td>
<td>0.66±0.34</td>
<td>0.52±0.25**</td>
</tr>
<tr>
<td>Delta-6 omega-6 (C18:3n-6/C18:2n-6)</td>
<td>0.016±0.007</td>
<td>0.016±0.008</td>
</tr>
<tr>
<td>Delta-5 omega-6 (C20:4n-6/C20:3n-6)</td>
<td>3.94±1.22</td>
<td>3.95±1.45</td>
</tr>
<tr>
<td>Delta 6 omega-6 (C22:5n-6/C22:4n-6)</td>
<td>0.67±0.16</td>
<td>0.65±0.22</td>
</tr>
<tr>
<td>Elongase (C20:3n-6/C18:3n-6)</td>
<td>3.81±2.67</td>
<td>3.47±3.05</td>
</tr>
<tr>
<td>Elongase (C22:4n-6/C20:4n-6)</td>
<td>0.025±0.007</td>
<td>0.024±0.007</td>
</tr>
<tr>
<td>Delta-9 (C14:1n-5/C14:0)</td>
<td>0.052±0.032</td>
<td>0.103±0.114***</td>
</tr>
<tr>
<td>Delta-9 (C16:1n-7/C16:0)</td>
<td>0.02±0.003</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td>Delta-9 (C18:1n-9/C18:0)</td>
<td>2.64±0.55</td>
<td>3.10±0.77**</td>
</tr>
<tr>
<td>Delta-9 (C24:1n-9/C24:0)</td>
<td>1.714±0.485</td>
<td>0.529±0.380**</td>
</tr>
<tr>
<td>Elongase (18:1 n-9/C16:1 n-9)</td>
<td>54.20±47.88</td>
<td>55.02±64.23</td>
</tr>
</tbody>
</table>

* Table is continued on the next page >
Elongase (C20:1 n-9/C18:1 n-9) 0.007±0.002 0.007±0.006
Elongase (22:1 n-9/C20:1 n-9) 0.90±0.71 0.33±0.61***
Elongase (C24:1 n-9/C22:1 n-9) 7.70±6.56 13.62±6.90***
Elongase (C18:1 n-7/C16:1 n-7) 0.77±0.25 0.65±0.28**
Elongase (C20:1 n-7/C18:1 n-7) 0.08±0.04 0.05±0.10*
Elongase (C16:0/C14:0) 20.62±6.26 18.26±6.54*
Elongase (C18:0/C16:0) 0.28±0.04 0.28±0.29
Elongase (C20:0/C18:0) 0.65±0.069 0.71±0.12***
Elongase (C22:0/C20:0) 2.32±0.36 1.97±0.57***
Elongase (C24:0/C22:0) 0.035±0.05 0.037±0.009*

\* Independent means t-tests: significantly different in comparison to controls at * $p<.05$, ** $p<.01$, *** $p<.001$.

In the ω-3 series the estimated activity of the elongase C22:5 ω-3/C20:5 ω-3 was significantly lower in the patients with MDD-R ($p<.01$). In the ω-9 series, the activity of the elongase C22:1 ω-9/ C20:1 ω-9 was significantly lower, but the activity of the next elongase C24:1 ω-9/ C22:1 ω-9 was significantly higher in the patients compared to controls ($p<.001$). The estimated activities of elongases in the ω-7 series (C18:1 ω-7/C16:1 ω-7 and C20:1 ω-7/C18:1 ω-7) were significantly lower in the patients. The estimated activity of elongase C22:0/C20:0 was significantly lower and the elongase C24:0/C22:0 significantly higher in the patients ($p<.001$).

Adjustment for waist circumference (Table 8)

Adjustment for WC as a characteristic of the Metabolic Syndrome (MetS), did not alter the significance of the differences between the concentrations of the FAs in the erythrocytes of the patients compared to the controls [We divided the p-values in four categories ($p\geq0.05$, $p<0.05$, $p<0.01$, $p<0.001$); no altering of significance means the p-value remained in the same category]. In plasma, the significance was altered for the differences in the concentrations of the following plasma FAs [C14:0, C16:0, C20:0, C16:1 ω-7, C18:1 ω-7, C16:1 ω-9, C18:1 ω-9 and C18:3 ω-3 (ALA)] between patients with MDD-R and controls (Table 8).
Table 8: Plasma fatty acid concentrations (µmol/l) of MDD-R patients compared with a matched non-depressed control group, with and without correction for waist circumferencea

<table>
<thead>
<tr>
<th></th>
<th>Controlsb (n = 65)</th>
<th>MDD-Rb (n = 137)</th>
<th>Controls (n = 65)</th>
<th>MDD-R (n = 137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linolenic acid (C18:3 ω-3)</td>
<td>67.38±5.6</td>
<td>77.22±3.9</td>
<td>63±40</td>
<td>79±45*</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1 ω-7)</td>
<td>267.3±29.7</td>
<td>375.2±20.6**</td>
<td>242±124</td>
<td>388±280***</td>
</tr>
<tr>
<td>Vaccenic acid (C18:1 ω-7)</td>
<td>169.5±7.32</td>
<td>195.6±5.08**</td>
<td>163±46</td>
<td>198±63***</td>
</tr>
<tr>
<td>Hypogaeic acid (C16:1 ω-9)</td>
<td>43.70±2.63</td>
<td>54.43±1.82**</td>
<td>42±15</td>
<td>55±23***</td>
</tr>
<tr>
<td>Oleic acid (C18:1 ω-9)</td>
<td>2086±116.5</td>
<td>2389±80.80*</td>
<td>1975±722</td>
<td>2439±1033***</td>
</tr>
<tr>
<td>Myristic acid (C14:0)</td>
<td>157.4±17.9</td>
<td>207.1±12.4</td>
<td>146±74</td>
<td>213±163***</td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>2794±138.1</td>
<td>3105±95.76</td>
<td>2651±707</td>
<td>3163±1271***</td>
</tr>
<tr>
<td>Arachidic acid (C20:0)</td>
<td>26.2±0.96</td>
<td>28.3±0.66</td>
<td>25.5±6.1</td>
<td>28.5±7.9**</td>
</tr>
</tbody>
</table>

a ANCOVA’s with correction for WC on the left and independent means t-tests on the right: significantly different in comparison to controls at * p<.05, ** p<.01, *** p<.001.
b WC corrected values.

Influence of anti-depressant use
To explore whether use of antidepressants was associated with different FA patterns we performed explorative ANOVA’s, comparing three groups of patients: 1) those who had used antidepressants continuously, 2) intermittent or 3) not at all. No differences in FA concentrations between these groups were found, except for C22:5 ω-3 in plasma (resp. 30.94 vs. 27.47 vs. 34.26, p<0.05) and C18:3 ω-3 (0.97 vs. 0.75 vs. 0.83, p<0.05) and 20:5 ω-3 (3.99 vs. 2.92 vs. 3.29, p<0.05) in erythrocyte membranes.

Influence of current depressive status
In the group of patients with recurrent depression, and FA concentrations of the patients with a current depression (n = 26) were compared with those of the non-depressed (n = 110) using explorative t-tests. The results showed no significant differences in mean plasma and erythrocyte FA concentrations between the two groups.

Influence of education level
After correction for differences in educational level (low, middle, high), the difference in C18:3 ω-3 in plasma between patients and controls, disappeared (67±6.3 vs. 78±4.2, p = .164). The other FAs in plasma and erythrocytes were not altered.
Discussion

Previous studies generally focussed on ω-3 and ω-6 PUFAs in samples of patients with a single depressive episode or high levels of depressive symptoms \(^4,5,7\). In the present study we were able to compare not only PUFA concentrations of the ω-3 and ω-6 series, but also ω-5, ω-7 and ω-9 MUFAs, as well as SFAs, in plasma and erythrocytes of patients with the recurrent type of MDD with those of matched control subjects.

The results of the present study confirm the results of our explorative pilot study \(^18\). Most striking was the finding that the concentrations of plasma and erythrocyte MUFAs and SFAs and additionally erythrocyte PUFAs, with a chain length >20 C atoms were all lower in the patients with MDD-R with the exception of C24:0 in the plasma and C22:1 ω-9 in the erythrocyte which did nor differ in patients and controls. In contrast, the concentrations of most of the shorter chain members (≤18 C) of all these families were higher in patients, with the exception of lower concentrations of C14:1 ω-5 and C16:1 ω-9 in erythrocytes.

Plasma FA levels reflect the combined effect of recent intake and endogenous processing \(^16\). So the increase in SFAs and MUFAs with ≤18 C in the patients may reflect the effect of a higher intake whether or not associated with higher activity of Δ9 desaturases. The estimated activities of the Δ9 desaturases C14:0/C14:1 ω-5 and C18:0/C18:1 ω-9 in the plasma were significantly higher in the depressive patients (Table 7). Enhanced Δ-9 desaturase activity has also been reported in patients with the MetS \(^12\).

The Σ ω-6 PUFAs in plasma was significantly higher in the patients than in the controls consistent with a higher intake of mainly LA. However, in contrast to many earlier studies, we found that levels of AA, EPA, DHA and the Σ ω-3 PUFAs in plasma were similar in patients and controls, as were AA/EPA and AA/DHA ratios \(^4,5,7\). Also estimated activities of Δ5 and Δ6 desaturases did not differ.

In the erythrocytes of our MDD-R patients the sums of ω-3 and the ω-6 PUFAs were lower and the ratios Σ ω-6/Σ ω-3 were higher than in controls corresponding with many reported data \(^4,5,7\). But as in plasma, the ratios AA/EPA and AA/DHA did not differ between patients and controls. The increase in the Δ6 desaturase products C18:4 ω-3 and C18:3 ω-6 might be compensatory to the decreases in C22:5 ω-3, C22:6 ω-3 and C20:3 ω-6 up to C22:5 ω-6. These alterations are also seen in the MetS \(^14,22\).
So, part of our most marked findings may be explained by the fact that based on plasma levels, the patients with MDD-R consume more SFAs and MUFAs and ω-6 PUFAs, C18:2 ω-6 (LA) in particular. The increase in the levels of C22:2 ω-6 (docosadienoic acid) in plasma and erythrocytes of the patients may also be explained thereby 23.

To our knowledge, concentrations of the longer chain FAs (≥20-24 C) of SFAs and MUFAs and estimates of their elongases were not systematically addressed in patients with MDD. We found a significant decrease of the >20 C plasma SFAs and MUFAs and of all the >20 C erythrocyte FA levels, with the exception of plasma C24:0 and erythrocyte C22:1 ω-9 which were similar in patients and controls.

One explanation may be altered elongase activity (Table 7). Interestingly, our results show decreased elongase activity on 20C FAs, whereas elongases acting on 22C FAs show increased activity, with the exception of the ω-6 series. However, the use of FA ratio’s for enzyme activity estimation gives only “surrogate” measures and may not reflect real activity 16.

Although not often reported, VLC SFAs and MUFAs, mainly C24:0 (lignoceric acid) and C24:1 ω-9 comprise 1-6% of total plasma PLs. These fatty acids are very abundant (up to 46 mol%) in sphingomyelin. In lower concentration (approximately 0.11-0.015) are FAs with 26 carbons such as C26:0 and C26:1 ω-9 and C26:2 ω-6. These fatty acids are also typically found in the sphingomyelin fraction 16.

We would like to hypothesize that the decreases in the longer chain FAs could be related to the use of these longer chain FAs for increased ceramide and sphingolipid synthesis as seen in insulin resistance and MetS 24. Furthermore, these long chain SFAs could be used for production of C26:0. High levels of C26:0 in whole blood were recently shown to be associated with the MetS in Japanese men 25.

Taken together, the cause of the decrease in concentrations of plasma and erythrocyte MUFAs and SFAs and additionally erythrocyte PUFAs, all with a chain length of ≥20 C atoms may well be multifactorial, that is to say modulated by dietary factors, life style, decreased or impaired activity of their respective desaturases and elongases, but could also correspond with decreased or increased incorporation in and/or enhanced detachment from the various plasma lipid components and/or cell membranes. Further research is needed to differentiate between these possibilities.
Consistent with the literature on obesity in depressive disorders, BMI, WHR and WC were all significantly higher in our MDD-R patients than in the control subjects. The MetS criterion of an increased WC (≥88 cm in women and ≥102 cm in men) was met in 44 of the 102 female patients (mean 87; range 63-122). Adjustment for WC, as a peripheral indicator of insulin resistance associated with visceral fat, resulted in reduced significances of the differences in most of the short chain members of the FA series in plasma (Table 8). This may correspond with the presence of the MetS and/or associated factors such as the dietary composition. However, the differences found in the longer chain plasma FAs and all differences in the erythrocyte were not altered by adjustment for WC. So, our main finding of lower concentrations of the longer members of the various FA series can not merely be explained by the presence of the MetS, and may reflect other MDD-R associated factors.

In this study after correction for differences in educational level (low, middle, high), the difference in C18:3 ω-3 in plasma between patients and controls disappeared, but the other FAs in plasma and erythrocytes were not altered. The percentage of smokers vs. non-smokers did not differ between patients and controls and so are not likely to be responsible for the differences in FA concentrations.

Antidepressant (AD) use was accompanied by altered concentrations of a small number of ω-3 PUFAs (C18:3 ω-3 and C20:3 ω-3 in plasma and C22:5 ω-3 in the erythrocyte membrane), other FA concentrations were not altered. There may be several explanations such as: the presence of a more serious depression in the AD users, the consumption of more food and less physical activity as well as an effect of AD on FA metabolism and (oxidative) stress. To our knowledge data of the direct effects of AD on FAs are still lacking and reports on the effect of AD on oxidative stress are sparse and still inconsistent. Our study was not directed at these aspects so we refrain from drawing conclusions.

We found no influence of current depressive status on FA concentrations, so they seem to be state-independent. This may indicate that the FA alterations in this study could represent a biological “trait” marker for recurrent depression (MDD-R).

An essential characteristic of MDD-R is the presence of increased oxidative stress, which may also modulate FA alterations in our patients. Oxidative stress is the result of an imbalance between excessive free radical oxygen species (ROS) production and/or diminished anti-oxidant defence mechanisms. Evidence for a causal role of oxidative stress in the pathogenesis of
psychiatric diseases including bipolar disorder and depression is steadily accumulating. The brain is particularly vulnerable because of its high oxygen consumption and hence generation of ROS combined with a high PUFA content and modest antioxidant defences.

Enhanced oxidative stress in our patients may be the result of a cumulative effect of the presence of the MetS and depression associated factors such as: psychological stress, hypothalamic-pituitary-adrenal (HPA)-axis hyperactivity, lifestyle changes (ω-3 PUFA deficient diet, physical inactivity, alcohol abuse and smoking). Major depressive disorder -its recurrent, chronic form in particular- may be accompanied by sustained activation of the HPA-axis.

Moreover, mitochondria are the principal ROS producers and evidence for genetically determined mitochondrial dysfunction in psychiatric disease, MDD included, is also growing steadily. Mitochondrial dysfunction will further enlarge increased ROS production and may also explain the increased prevalence of the MetS and CVD in MDD-R.

Noteworthy, the pattern of FA alterations in our patients are not specific for MDD-R but is also found in other (psychiatric) diseases accompanied by increased oxidative stress e.g. bipolar disorder, schizophrenia, diabetes, Alzheimer’s disease and are also seen during normal aging.

Finally, the FA alterations in MDD-R could fulfill an adaptive or protective role, as the alterations may render cell membranes less vulnerable for oxidation. SFAs and MUFAs are more resistant to oxidative stress, while the more polyunsaturated a FA, the more susceptible it is.

Our study has several distinct limitations: dietary intake of fat and FAs, alcohol consumption, smoking habits, physical activity and the presence of the MetS were not systematically assessed. We used ratios for estimating desaturases and elongases and did not measure enzyme expression/activities. However, in spite of these many limitations, our results were quite consistent which may argue for their validity. This consistency, together with the magnitude of our findings, also makes type I errors -that could have occurred because of multiple testing- less likely.

At this moment the basal question; whether fatty acids alterations in depressive patients are either the cause or the consequence of the disease, cannot yet be answered on the basis of available studies (ecologic, observational, and RCTs). They provide inconsistent data, the very
few prospective studies included \(^{39,40}\). In their most recent review, Appleton et al. stressed that in relation to the effects of \(\omega-3\) PUFAs, an important distinction may exist between diagnosed depressive illness and the less severe, undiagnosed, or precursor “depressed mood” \(^{40}\).

The best methods to study FAs have not yet crystallized. The FA composition of the different blood lipid fractions, e.g. plasma, erythrocytes and platelets is inter-related, particularly for PLs. As erythrocytes have a life span of approximately 120 days, large changes that occur within days of altering dietary fat intake can only be explained by exchange and transfer of FA from plasma to erythrocytes \(^{16}\).

Although erythrocyte FA composition is comparable to that of plasma total PLs, there are differences \(^{16}\). Given these differences it will prove to be more informative to analyse FAs in both compartments. For prospective studies over several years FA biomarkers from erythrocytes or adipose tissue, which reflect longer-term intakes (preceding months or years, respectively) would be more suitable to test the association between long term PUFA status and depression \(^{39,40}\).

Adequately powered intervention studies are urgently needed, studying the effect dependent on the background FA status and the change during the study period. Further FA research should include: dietary questionnaires, analysis of the complete FA spectrum and also the enzymes involved in their metabolism. It is increasingly demonstrated that polymorphisms in the genes/enzymes (desaturases, elongases) that regulate biosynthesis of EPA, DHA and AA from their precursors (ALA, EPA) may represent important determinants of plasma and tissue PUFA levels \(^{41}\). Measurement of (oxidative) stress levels and lipid peroxidation products and assessment of MetS symptoms will help us to distinguish between harmful or adaptive FA changes. It will also help us to determine whether patients will benefit from anti-oxidant strategies and/or any form of FA supplementation.

**Acknowledgments**

We are most grateful to the participants of our study. In addition, we express our appreciation to the participating psychiatric sites for their recruitment efforts. We also thank our interviewers and independent raters and specifically Irene Visch for assistance with data management and support. The following colleagues contributed to the DELTA (Depression Evaluation Longitudinal Therapy Assessment) Study: Mascha ten Doesschate, Jochanan Huyser, Maarten Koeter, Guido Nabarro, Philip Spinhoven, Ellie Wekking en Luuk Wouters.
Competing Interests
The authors have declared that no competing interests exist.

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Fatty acid metabolism

4.3 Ala54Thr fatty acid-binding protein 2 (FABP2) polymorphism in recurrent depression: associations with fatty acid concentrations and waist circumference

Mocking RJT *, Lok A *, Assies J, Koeter MWJ, Visser I, Ruhé HG, Bockting CLH, Schene AH

* Authors contributed equally

Submitted for publication
Abstract

Background
Fatty acid (FA)-alterations may mediate the mutual association between Major Depressive Disorder (MDD) and cardiovascular disease (CVD). However, etiology of observed FA-alterations in MDD and CVD remains largely unclear. An interesting candidate may be a mutation in the fatty acid-binding protein 2 (FABP2)-gene, because it regulates dietary FA-uptake. Therefore, we aimed to test the hypotheses that in MDD-patients the FABP2 Ala54Thr-polymorphism would be (I) more prevalent than in sex- and age-matched controls, (II) associated with observed alterations in FA-metabolism, and (III) associated with CVD-risk factor waist circumference.

Methods
We measured concentrations of 29 different erythrocyte FAs, FABP2-genotype, and waist circumference in recurrent MDD-patients and matched never-depressed controls.

Results
FABP2-genotype distribution did not significantly differ between the 137 MDD-patients and 73 matched controls. However, patients with the Ala54Thr-polymorphism had (I) higher concentrations of especially eicosadienoic acid (C20:2ω6; P=.009) and other 20-carbon FAs, and associated (II) lower waist circumference (P=.019). In addition, FABP2-genotype effects on waist circumference in patients seemed (I) mediated by its effect on C20:2ω6, and (II) different from controls.

Conclusions
Although Ala54Thr-polymorphism distribution was not associated with recurrent MDD, our results indicate that FABP2 may play a role in the explanation of observed FA-alterations in MDD. For Ala54Thr-polymorphism patients, potentially adaptive conversion of increased bioavailable dietary precursors into eicosadienoic acid instead of arachidonic acid might be related to a low waist circumference. Because this is the first investigation of these associations, replication is warranted, preferably by nutrigenetic studies applying lipidomics and detailed dietary assessment.
1. Introduction

Major depressive disorder (MDD) - in particular its recurrent form (MDD-R) - is a major cause of disability and excess mortality worldwide. The leading cause of this excess mortality in MDD is cardiovascular disease (CVD). Accordingly, the primary CVD-risk factor waist circumference - reflecting abdominal obesity and insulin resistance - is strongly associated with MDD.

Improved understanding of mechanisms underlying the MDD-CVD relationship might lead to novel life-prolonging (preventive) treatment strategies.

An important mechanism underlying the MDD-CVD relationship may be fatty acid (FA)-metabolism, because it regulates e.g. inflammation, thrombosis and neurotransmitter-signaling. FA-composition of cell membranes is altered in both MDD and CVD, with decreased ω3 polyunsaturated fatty acids (PUFAs) and increased ω6/ω3 PUFA ratios, e.g. increased arachidonic acid (C20:4ω6; ARA) relative to eicosapentaenoic acid (C20:5ω3; EPA). We corroborated these findings in a sample of 137 MDD-R patients, and extended them by showing additional changes in other FA-classes, e.g. lower overall FA-unsaturation, -chain length and -peroxidation. However, etiology of FA-alterations in MDD and CVD remains largely unclear.

FA-metabolism is influenced by many different factors, including genotype, dietary intake (e.g. essential ω3 FAs from fatty fish), lifestyle (physical activity, smoking), and hormonal regulation. Interestingly, we previously reported that FA-alterations in MDD-R follow a bimodal distribution. Instead of being unimodally distributed, with regard to FA-alterations, MDD-R-patients seemed to consist of two separate groups reflected by two unimodal distributions, a phenomenon also observed in schizophrenia. This implies a dichotomous causal factor that divides patients in two groups and thereby underlies these bimodally distributed FA-alterations. A possible example of such a dichotomous causal factor may be a mutation in a gene involved in FA-metabolism.

An interesting location for such a mutation may be the fatty acid-binding protein 2 (FABP2) gene. FABP2 is mainly expressed in small intestine enterocytes, where it is responsible for uptake of dietary FAs. A transition G to A at FABP2-codon 54 results in an alanine (Ala) to threonine (Thr) amino acid substitution (Ala54 to Thr54). This single nucleotide polymorphism is common, with a Thr54 allelic frequency of 30% in most populations, resulting in altered FABP2 FA-affinity. Homozygous Thr54-carriers show altered dietary FA-uptake, with increased postprandial concentrations of 14-18-carbon fatty acids. Because of the (patho)physiological role of FAs in metabolism, this altered FA-uptake has been suggested to
explain the association of FABP2 with increased insulin resistance and FA-oxidation, supporting observations suggesting a role of the FABP2 Ala54Thr-polymorphism in CVD-etioloogy (e.g. increased waist circumference and atherosclerosis) 21, 22, 23, 24, 25.

Considering this involvement of FABP2 in FA-metabolism, the FABP2 Ala54Thr-polymorphism may also be particularly interesting in the explanation of FA-metabolism alterations in MDD-R patients, because - as stated above - we observed (I) increased concentrations of 14-18-carbon FAs of several FA-subclasses 14, and (II) highly significantly lower overall FA-chain length [15], which was (III) bimodally distributed 18. Surprisingly, previous studies in healthy and CVD-populations found no consistent associations of FA-concentrations with the FABP2 Ala54Thr-polymorphism 24, 26, 27. This may be because, to our knowledge, not all members of the different FA-subclasses (e.g. long chain saturated and monounsaturated FA’s) were studied, especially not in a specific psychiatric (e.g. depressed) population with known bimodally distributed FA-alterations.

Therefore, the aim of the present study was to test the hypotheses that in MDD-R-patients the Thr54-polymorphism in the FABP2-gene would be (I) more prevalent than in sex-and age-matched controls, (II) associated with observed (bimodally distributed) alterations in FA-metabolism, and (III) associated with CVD-risk factor waist circumference.

2. Methods and materials
2.1. Ethics Statement
All participants provided written informed consent prior to enrolment. The ethics committee of the Academic Medical Center of the University of Amsterdam approved the study.

2.2 Subjects
The current study was part of the DELTA-study, a randomized clinical trial on the effect of an 8-week cognitive therapy on MDD-recurrence, described previously 28, 29. This trial has been registered in the ISRCTN registry as ISRCTN68246470. As part of DELTA-study’s 2-year follow-up measurements, we invited subjects to participate in the current study. At the start of the DELTA-study, participants had to (I) be aged between 18 and 65, (II) have had ≥2 previous major depressive episodes in the last five years, and (III) be in remission of MDD. Exclusion criteria were current or previous mania or hypomania, any psychotic disorder, alcohol or drug abuse, and predominant anxiety disorder. Since any form of therapy (e.g. antidepressant-use) was no inclusion or exclusion criterion for the trial, the DELTA-sample can be considered representative for MDD-R patients with respect to this characteristic.
In addition to the MDD-R patient sample, we recruited age- and gender-matched healthy control subjects, without a personal and/or family history of MDD, as described previously.

2.3 Measurements
We took 20 ml blood by venipuncture from subjects in the nonfasting state. As a model of brain FA-concentrations, we used washed erythrocytes, stored at -80 °C until analyses by capillary gas chromatography, as described previously. This resulted in data on 29 different FAs, expressed in pmol/10^6 erythrocytes. We operationalized FA-metabolism in two steps. First we tested five main FAs: linoleic acid, arachidonic acid, α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid, together with three indices which describe overall FA-characteristics: the Unsaturation (UI), Peroxidation (PI) and Chain Length Indices (CLI).

Subsequently, we exploratively tested 24 other FAs. This approach reduces the multiple testing problem, because it guides interpretation of effects as explorative or a priori selected.

DNA was isolated from blood using a filter-based method (QIAamp DNA Mini Kit, Qiagen Ltd, United Kingdom). Polymerase chain reaction (PCR) primers were designed using Primer 3. PCR primer sequence TGACAATTTGAAGCTGACAATTA and AATCAAGAATGCATTGCTCAT, PEX primer sequence bioAT AAA TTC ACA GTC (L)AA GAA TCA AGC. Genotyping was done using a Matrix Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) mass spectrometer from Bruker Daltonics. All samples were genotyped in duplicate to increase reliability. Genotyping error rate based on these duplicates was 3.7%. We operationalized FABP2-genotype in three categories: GG homozygous, AG heterozygous and AA homozygous. An A-allele results in the Thr54-polymorphism.

We determined waist circumference in centimeters using a standard operating procedure.

2.4 Statistical analyses
2.4.1 Missing data
We used multiple imputation to prevent bias possibly introduced by missing or non-detectable FA- or genotype-data, which resulted in 5 imputed datasets as described previously.

2.4.2 Hardy-Weinberg Equilibrium (HWE)
We tested deviations from HWE separately in patients and controls using an online calculator χ²-test, available on http://www.ogee.org/software/hwe-mr-calc.html.
2.4.3 Subject characteristics
We compared patients’ and controls’ subject characteristics using χ²-tests or independent Student’s t-tests as appropriate.

2.4.4 Hypotheses testing
For the first hypothesis, we used χ²-tests to test whether the distribution of observed genotype frequencies for patients and controls differed from expected frequencies. In order to test the association of the Thr54-polymorphism with the bimodally distributed FA-alterations in MDD-R-patients (2nd hypothesis), we used linear mixed models with genotype as predictor variable and a FA-concentration as outcome variable. In case the overall F-test for a given FA was significant, we reported parameter estimates for the distinct genotypes with GG as reference category. For the association of Thr54-polymorphism with CVD-risk (3rd hypothesis), we applied a similar model, except that we entered waist circumference as outcome variable. We performed no correction for confounders, because these effects concern genetic effects and genotype is not expected to be subject to confounding factors.

In addition, we planned several post-hoc tests. First, in case FA-alterations would show linear associations with FABP2 genotype, e.g. GG: lowest value, AG: middle value, AA, highest value, we would test the effect of FABP2 on FA-alterations in linear mixed models with FABP2 recoded as scale variable as predictor variable and the FAs as outcome variable. Second, if FA-concentrations and waist circumference would be associated with FABP2-genotype, we would test correlations between these FA-alterations and waist circumference. If these alterations would be significantly correlated, in order to distinguish the direction of these effects, we would perform mediation analyses. To this end, we would use linear mixed models, first with FABP2 and waist circumference as predictor variables and the FA as outcome variable, subsequently with FABP2 and the FA as predictor variables and waist circumference as outcome variable. If the effect of FABP2 on the FA or waist circumference would disappear after inclusion of the other factor (the FA or waist circumference) in the model, this would imply that this other factor mediates the influence of FABP2. Finally, third, if FABP2 would affect FA-concentrations or waist circumference in the MDD-patients, we would test whether this effect would differ from the effect in the matched controls. In order to test this, we would build another set of linear mixed models with FABP2 and patients-status (patient or control) and their interaction (FABP2×patient-status) as predictor variables and the FA or waist circumference as outcome variable. Because the patient-status factor in these models may be subject to confounding, in contrast to the genetic FABP2-factor, we corrected observed effects for possibly confounding differences.
between patients and controls in age, sex, marital status, educational level, social class, ethnicity, 17-item Hamilton depression rating scale (HDRS)-score, and smoking. To prevent losing power, we used propensity scores, which enable correction for multiple potentially confounding factors while retaining power. Propensity scores represent for each case the saved predicted probability of being a patient or a control, which we calculated using a binary logistic model with patient-status as dependent, and the chosen potential confounders as predictor variables.

Although often not dealt with in FA-research, the multitude of FAs makes multiple comparisons inherent in investigating FA-metabolism, potentially causing type-I errors \(^{15}\). To reduce this problem we (I) applied pathophysiologically driven data reduction using the UI, CLI and PI \(^{16}\), and (II) a priori designated several outcomes as hypothesis based, and others as explorative. Although still subject to debate, Bender and Lange \(^{31}\) suggest to perform correction for multiple testing primarily in confirmatory studies, while explorative results should be clearly indicated as such \(^{31}\). In line with their advice, and because e.g. Bonferroni correction likely would be too strict considering the relatively strong assumed correlations between the different outcomes in this study and therefore may induce type-II errors, we consequently chose to correct neither the results of the a priori hypothesized outcomes, nor the explorative results, for multiple comparisons \(^{31}\). Therefore, particularly the explorative results of this first investigation of associations between FABP2 and several FAs - particularly in a psychiatric population - should be interpreted as such.

2.4.5. Power analyses
We performed sensitivity power analyses. With power=.80 and two-sided alpha=.05, we were able to detect effects with a small effect size (\(w>0.214; f>0.216; f^2>0.080\)) for all analyses, except for the differences between the different genotype classes in FA-measures and waist circumference in the patient group, for which we were able to detect medium effect sizes (\(f>0.268\)).

2.4.6. Software
We performed multiple imputation using the package Amelia II, implemented in R \(^{32}\). We performed analyses using SPSS Statistics v.20 (IBM). For multiple imputation results that are not pooled by SPSS, we used Rubin’s rules \(^{32}\). For the linear mixed models, these were implemented in a macro available at http://fswweb07.fsw.leidenuniv.nl/Docs/MI-mul2.zip, as described previously. We used G*Power 3.1.3 (Kiel, Germany) to perform power calculations.
3. Results
3.1 Included sample and missing data
The inclusion procedure resulted in 137 patient and 73 control participants. Of these subjects, 8 patients and 3 controls had no valid \textit{FABP2}-genotype due to technical reasons. Missingness in FA-data has been described previously \cite{15}. Standard diagnostics by Amelia II suggested successful imputation.

3.2 Subject characteristics
\textit{FABP2}-genotype was in HWE in controls (\(P>0.05\)). Equal sex and age distributions among patients and controls indicate successful matching (\(P>0.05\)). Patients had lower educational level (\(P<0.001\)) and greater waist circumference (\(P=0.025\)) (Supplementary Table).

Supplementary table: Subject Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients</th>
<th>Controls</th>
<th>(p) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((n = 137))</td>
<td>((n = 73))</td>
<td>\</td>
</tr>
<tr>
<td>Female, %</td>
<td>73.7%</td>
<td>69.9%</td>
<td>.55</td>
</tr>
<tr>
<td>Age, mean (SE), year</td>
<td>46.4 (0.8)</td>
<td>44.7 (1.1)</td>
<td>.205</td>
</tr>
<tr>
<td>Educational level(a), %</td>
<td>&lt;.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>33.3</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>31.2</td>
<td>22.5</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>35.5</td>
<td>72.3</td>
<td></td>
</tr>
<tr>
<td>Social class(b), %</td>
<td>&lt;.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 1</td>
<td>11.1</td>
<td>55.0</td>
<td></td>
</tr>
<tr>
<td>Class 2</td>
<td>52.1</td>
<td>32.0</td>
<td></td>
</tr>
<tr>
<td>Class 3</td>
<td>36.7</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td>Smoking, %</td>
<td>49.0</td>
<td>39.4</td>
<td>.21</td>
</tr>
<tr>
<td>Waist circumference, mean (SE), cm</td>
<td>89.3 (1.19)</td>
<td>84.9 (1.42)</td>
<td>.025</td>
</tr>
<tr>
<td>HDRS17 score, mean (SE)</td>
<td>5.9 (.46)</td>
<td>1.2 (.48)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Current depressive episode, % (n)</td>
<td>19.0 (26)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Antidepressant use, % (n)</td>
<td>62.8 (86)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>TCA, % (n)</td>
<td>5.1 (7)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>SSRI, % (n)</td>
<td>38.7 (53)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Other, % (n)</td>
<td>19.0 (26)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Number of previous episodes, mean (SE)</td>
<td>7.71 (0.76)</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

\textit{Table is continued on the next page >}
**Time after Time: biological factors in the course of recurrent depression**

**Age of first onset, mean (SE), year** | 28.4 (1.08) | NA  
**SSRI, % (n)** | 38.7 (53) | NA  
**Other, % (n)** | 19.0 (26) | NA  
**Number of previous episodes, mean (SE)** | 7.71 (0.76) | NA  
**Age of first onset, mean (SE), year** | 28.4 (1.08) | NA  

**Abbreviations** - HDRS = Hamilton depression rating scale; TCA = tricyclic antidepressant; SSRI = selective serotonin reuptake inhibitor.

*a* Educational level is defined as: low, primary education or preparatory middle-level applied education; middle, higher general continued education or middle-level applied education; and high, preparatory scientific education, higher applied education, or scientific education.

*b* based on occupation: Class 1, e.g. cleaner; Class 2, e.g. nurse; Class 3, e.g. general manager.

### 3.3 FABP2-distribution in patients and controls

The distribution of FABP2-genotype did not differ among patients and controls (*P*=.627). In patients, genotype frequencies were GG: 57.7%, AG: 34.3%, and AA: 8.0%, and in controls 50.7%, 41.6%, and 7.7%, respectively.

### 3.4 Association FABP2-FA-concentrations

Regarding the association of the Thr54-polymorphism with FA-metabolism, MDD-R patients’ FA-concentrations of the a priori selected five main FAs and three indices did not differ according to FABP2-genotype (Table 1). In the explorative analyses of the other FAs, two FA-concentrations were significantly associated with FABP2-genotype: eicosadienoic acid (C20:2ω6; *F*₂,₂₂₂.₆ =4.801,*P*=.009) and docosadienoic acid (C22:2ω6; *F*₂,₂₈₉.₁ =3.271,*P*=.039; Table 1). Looking at the parameter estimates for the three subgroups, the AA-genotype had higher eicosadienoic acid (*b*=0.437, *SE*ₚ=0.156, *t*=7.886,*P*=.006) and docosadienoic acid-concentrations (*b*=0.236, *SE*ₚ=0.120, *t*=2.324,*P*=.020) compared to the reference GG-category (Figure 1).

### 3.5 Association FABP2-waist circumference

Considering the third hypothesis, MDD-R patients’ waist circumference significantly differed according to FABP2-genotype (*F*₂,₈₉₆.₆ =4.000,*P*=.019). AA-genotype was associated with lower waist circumference estimate compared to the reference GG category (*b*=−12.243, *SE*ₚ=4.474, *t*=−2.737,*P*=.006; Figure 2, Panel A).
Table 1: Differences in fatty acid concentrations, fatty acid indices and waist circumference between GG, AG and AA-carriers of the Ala54Thr fatty acid-binding protein 2 (FABP2) polymorphism, vertically graphically divided in a priori (upper panel) and explorative (lower panel) tests

<table>
<thead>
<tr>
<th></th>
<th>GG</th>
<th>AG</th>
<th>AA</th>
<th>F-value</th>
<th>Df1</th>
<th>Df2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:2n6</td>
<td>65.42±1.528</td>
<td>67.05±1.922</td>
<td>65.64±4.198</td>
<td>0.215</td>
<td>2</td>
<td>87.502 .807</td>
<td></td>
</tr>
<tr>
<td>C20:4n6</td>
<td>71.07±0.976</td>
<td>73.51±1.374</td>
<td>71.78±2.819</td>
<td>1.003</td>
<td>2</td>
<td>44.068 .375</td>
<td></td>
</tr>
<tr>
<td>C18:3n3</td>
<td>0.826±0.036</td>
<td>0.845±0.044</td>
<td>0.848±0.091</td>
<td>0.073</td>
<td>2</td>
<td>163.23 .930</td>
<td></td>
</tr>
<tr>
<td>C20:5n3</td>
<td>3.381±0.188</td>
<td>3.278±0.270</td>
<td>3.381±0.534</td>
<td>0.049</td>
<td>2</td>
<td>56.873 .952</td>
<td></td>
</tr>
<tr>
<td>C22:6n3</td>
<td>15.19±0.558</td>
<td>14.63±0.844</td>
<td>14.16±1.535</td>
<td>0.287</td>
<td>2</td>
<td>38.681 .752</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1.101±0.012</td>
<td>1.096±0.016</td>
<td>1.107±0.031</td>
<td>0.057</td>
<td>2</td>
<td>106.47 .945</td>
<td></td>
</tr>
<tr>
<td>CLI</td>
<td>18.31±0.023</td>
<td>18.30±0.030</td>
<td>18.38±0.065</td>
<td>0.601</td>
<td>2</td>
<td>164.04 .549</td>
<td></td>
</tr>
<tr>
<td>UI</td>
<td>1.290±0.009</td>
<td>1.289±0.012</td>
<td>1.305±0.023</td>
<td>0.187</td>
<td>2</td>
<td>125.27 .830</td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>91.40±1.556</td>
<td>88.04±2.010</td>
<td>79.16±4.173</td>
<td>4.000</td>
<td>2</td>
<td>896.61 .019</td>
<td></td>
</tr>
<tr>
<td>C14</td>
<td>3.333±0.116</td>
<td>3.287±0.154</td>
<td>2.817±0.351</td>
<td>1.047</td>
<td>2</td>
<td>83.660 .355</td>
<td></td>
</tr>
<tr>
<td>C16</td>
<td>162.8±2.597</td>
<td>165.5±3.300</td>
<td>157.9±7.915</td>
<td>0.466</td>
<td>2</td>
<td>90.552 .629</td>
<td></td>
</tr>
<tr>
<td>C18</td>
<td>103.0±1.206</td>
<td>104.6±1.554</td>
<td>100.4±3.338</td>
<td>0.761</td>
<td>2</td>
<td>295.71 .468</td>
<td></td>
</tr>
<tr>
<td>C20</td>
<td>2.490±0.042</td>
<td>2.597±0.054</td>
<td>2.705±0.132</td>
<td>1.965</td>
<td>2</td>
<td>46.139 .152</td>
<td></td>
</tr>
<tr>
<td>C22</td>
<td>7.568±0.190</td>
<td>7.481±0.234</td>
<td>8.287±0.501</td>
<td>1.085</td>
<td>2</td>
<td>120.20 .341</td>
<td></td>
</tr>
<tr>
<td>C24</td>
<td>14.78±0.496</td>
<td>14.64±0.653</td>
<td>16.40±1.378</td>
<td>0.727</td>
<td>2</td>
<td>84.733 .486</td>
<td></td>
</tr>
<tr>
<td>C18:4n3</td>
<td>0.221±0.028</td>
<td>0.280±0.037</td>
<td>0.246±0.078</td>
<td>0.777</td>
<td>2</td>
<td>133.36 .462</td>
<td></td>
</tr>
<tr>
<td>C22:5n3</td>
<td>8.009±0.196</td>
<td>7.913±0.251</td>
<td>7.918±0.543</td>
<td>0.048</td>
<td>2</td>
<td>87.828 .953</td>
<td></td>
</tr>
<tr>
<td>C18:3n6</td>
<td>0.583±0.026</td>
<td>0.589±0.030</td>
<td>0.479±0.083</td>
<td>0.906</td>
<td>2</td>
<td>24.758 .417</td>
<td></td>
</tr>
<tr>
<td>C20:3n6</td>
<td>8.879±0.284</td>
<td>8.796±0.376</td>
<td>9.738±0.887</td>
<td>0.536</td>
<td>2</td>
<td>40.268 .589</td>
<td></td>
</tr>
<tr>
<td>C22:4n6</td>
<td>10.46±0.310</td>
<td>10.67±0.409</td>
<td>11.64±0.848</td>
<td>0.883</td>
<td>2</td>
<td>153.36 .416</td>
<td></td>
</tr>
<tr>
<td>C22:5n6</td>
<td>1.695±0.070</td>
<td>1.738±0.095</td>
<td>1.777±0.179</td>
<td>0.126</td>
<td>2</td>
<td>62.253 .882</td>
<td></td>
</tr>
<tr>
<td>C20:2n6</td>
<td>1.243±0.049</td>
<td>1.377±0.065</td>
<td>1.680±0.148</td>
<td>4.801</td>
<td>2</td>
<td>222.65 .009</td>
<td></td>
</tr>
<tr>
<td>C22:2n6</td>
<td>0.509±0.036</td>
<td>0.470±0.050</td>
<td>0.745±0.098</td>
<td>3.271</td>
<td>2</td>
<td>289.13 .039</td>
<td></td>
</tr>
<tr>
<td>C14:1n5</td>
<td>0.288±0.036</td>
<td>0.308±0.044</td>
<td>0.286±0.110</td>
<td>0.055</td>
<td>2</td>
<td>40.243 .947</td>
<td></td>
</tr>
<tr>
<td>C16:1n7</td>
<td>3.181±0.172</td>
<td>3.068±0.262</td>
<td>2.986±0.521</td>
<td>0.099</td>
<td>2</td>
<td>40.381 .906</td>
<td></td>
</tr>
<tr>
<td>C18:1n7</td>
<td>7.469±0.169</td>
<td>7.651±0.246</td>
<td>7.162±0.533</td>
<td>0.403</td>
<td>2</td>
<td>38.358 .671</td>
<td></td>
</tr>
<tr>
<td>C20:1n7</td>
<td>0.254±0.029</td>
<td>0.304±0.038</td>
<td>0.261±0.100</td>
<td>0.443</td>
<td>2</td>
<td>41.976 .645</td>
<td></td>
</tr>
<tr>
<td>C16:1n9</td>
<td>0.798±0.149</td>
<td>1.124±0.204</td>
<td>1.078±0.543</td>
<td>0.730</td>
<td>2</td>
<td>13.743 .500</td>
<td></td>
</tr>
<tr>
<td>C18:1n9</td>
<td>74.61±1.183</td>
<td>74.92±1.563</td>
<td>73.49±3.390</td>
<td>0.075</td>
<td>2</td>
<td>98.188 .927</td>
<td></td>
</tr>
<tr>
<td>C20:1n9</td>
<td>1.151±0.042</td>
<td>1.207±0.056</td>
<td>1.409±0.123</td>
<td>2.099</td>
<td>2</td>
<td>62.402 .131</td>
<td></td>
</tr>
</tbody>
</table>

Table is continued on the next page >
### Table 1

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>GG</th>
<th>AG</th>
<th>AA</th>
<th>p-value</th>
<th>t-value</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C22:1n9</td>
<td>1.792±0.241</td>
<td>2.042±0.317</td>
<td>2.010±0.652</td>
<td>0.219</td>
<td>2</td>
<td>1928.1</td>
<td>.803</td>
</tr>
<tr>
<td>C24:1n9</td>
<td>13.16±0.415</td>
<td>13.25±0.541</td>
<td>14.52±1.208</td>
<td>0.624</td>
<td>2</td>
<td>87.715</td>
<td>.538</td>
</tr>
<tr>
<td>C20:3n9</td>
<td>0.397±0.028</td>
<td>0.371±0.036</td>
<td>0.243±0.076</td>
<td>1.849</td>
<td>2</td>
<td>101.56</td>
<td>.163</td>
</tr>
<tr>
<td>Total FAs</td>
<td>585.5±6.571</td>
<td>593.3±8.685</td>
<td>573.3±19.72</td>
<td>0.527</td>
<td>2</td>
<td>984.82</td>
<td>.590</td>
</tr>
</tbody>
</table>

### Abbreviations

- PI = Peroxidation Index; CLI = Chain Length Index; UI = Unsaturation Index; WC = Waist circumference.

### Figure 1

Concentrations of four fatty acids [eicosadienoic acid (C20:2ω6), docosadienoic acid (C22:2ω6), arachidic acid (C20:0) and gondoic acid (C20:1ω9)] in 137 recurrently depressed patients according to Ala54Thr fatty acid-binding protein 2 (FABP2) polymorphism genotype (GG, AG, AA).
3.6 Post-hoc analyses

3.6.1 Tests for linear \textit{FABP2}-FA-associations

In the analyses of the association of the Thr54-polymerism with FA-metabolism, we observed that while overall effect of genotype was not significant, parameter estimates for specific genotypes on some FAs were significant ($P<.05$) and suggested a linear associations of FA-concentrations across \textit{FABP2} genotype, e.g. \textit{GG}: lowest value, \textit{AG}: middle value, \textit{AA}, highest value. This affected especially FAs with a 20-carbon chain length. We therefore tested this linear relationship using linear mixed models with \textit{FABP2}-genotype recoded as a scale variable (\textit{GG}=1, \textit{AG}=2, \textit{AA}=3). These models showed significant linear effects of \textit{FABP2}-genotype on C20 ($b=0.106, SE_b=0.052, t_{135}=2.032, P=.045$), C20:2$\omega$6 ($b=0.182, SE_b=0.062, t_{135}=2.944, P=.004$), and C20:1$\omega$9 ($b=0.099, SE_b=0.049, t_{135}=2.001, P=.046$; Figure 1).

3.6.2 Relationship \textit{FABP2}, FA-concentrations, and waist circumference

In order to disentangle \textit{FABP2}’s effects on FA-concentrations and waist circumference, we performed correlation-analyses between waist circumference and FA-concentrations, and mediation analyses concerning the effect of \textit{FABP2} on FA-concentrations and waist circumference. In line with the effects of \textit{FABP2}-genotype on both eicosadienoic acid and waist circumference, a post-hoc Pearson’s correlation test showed that eicosadienoic acid ($r=-.243, P=.005$) was significantly negatively correlated with waist circumference in patients. Subsequently,
in a linear mixed model with both FABP2-genotype and waist circumference as predictors of eicosadienoic acid, we observed that both FABP2 ($F_{2,317.47} = 3.285, P = .039, b_{AA \text{ vs} \ GG} = 0.362, SE_b = 0.155, t_{173.915} = 2.342, P = .020$) and waist circumference ($F_{1,3184.09} = 4.790, P = .029, b = -0.006, SE_b = 0.003, t_{3184.09} = -2.189, P = .029$) independently predicted eicosadienoic acid concentrations (overall $F_{3,799.79} = 4.890, P = .002$). However, the other way around, the predictive effect of FABP2 on waist circumference lost significance ($P = .102$) after including eicosadienoic acid as a [significant ($P = .031$)] predictor in the model. This indicates that FABP2's effect on waist circumference is mediated by its effect on eicosadienoic acid, i.e. FABP2's effect on waist circumference seems to consist of higher eicosadienoic acid concentrations in A-allele carriers which on their turn result in lower waist circumference.

3.6.3 Interactions between patient-status and FABP2 on C20:2ω6 and waist circumference
To test whether the relation between FABP2 and C20:2ω6 and waist circumference differed between patients and controls, we performed linear mixed models with patient-status (yes/no) and FABP2-genotype (GG/AG/AA) and their interaction as predictor variables, and C20:2ω6 or waist circumference as outcome variables. There was no patient-status×FABP2-genotype interaction for C20:2ω6 ($F_{2,882.78} = 1.069, P = .344$). For waist circumference, the interaction was also non-significant ($F_{2,640.047} = 2.055, P = .129$), but the patient×AA-genotype parameter estimate was significant at trend level ($b = -15.140, SE_b = 7.719, t_{3184.09} = -1.961, P = .051$), suggesting that in patients AA-genotype was associated with a relatively lower waist circumference compared to controls (Figure 2, Panel A and B). After correction - using propensity scores - for possibly confounding differences between patients and controls in e.g. educational level, social class, HDRS-score, and smoking, the overall genotype effect remained non-significant ($F_{2,477.338} = 2.129, P = .120$). However, the patient×AA-genotype parameter estimate now just gained significance ($b = -15.681, SE_b = 7.946, t_{242.59} = -1.974, P = .050$).

4. Discussion
4.1 Summary of results
In the present paper, we examined FABP2's role in MDD-R, and particularly its relation with bimodally distributed FA-alterations and CVD-risk factor waist circumference. FABP2 Ala-54Thr-polymorphism distribution did not differ between 137 patients with recurrent MDD and 73 matched healthy controls without an MDD-history. However, A-allele carrying MDD-R patients had (I) higher concentrations of several 20-carbon FAs, especially eicosadienoic acid (C20:2ω6), and associated (II) lower waist circumference. In addition, post-hoc analyses suggested that ef-
fects of \textit{FABP2} on waist circumference in patients (I) were mediated by its effect on C20:2\omega 6, and (II) were different from controls.

4.2 \textit{FABP2} genotype distribution
Although it is increasingly recognized that FA-metabolism may play an important role in psychiatric disease, this is - to the best of our knowledge - the first investigation of the role of \textit{FABP2} in a psychiatric population. Contrary to our first hypothesis, genotype distribution did not differ from matched controls. This may imply that the Ala54Thr-polymerorphism plays no role in vulnerability for MDD-R, as opposed to what have been suggested for other disorders including obesity and atherosclerosis \cite{21, 22, 23, 24, 25}.

4.3 Association between \textit{FABP2} and FA-metabolism
However, with regard to our second hypothesis, although the earlier observed bimodally distributed FA-metabolism parameters \cite{18} were not associated with the Ala54Thr-polymerorphism, we observed specific associations of the A-allele with the biologically mutually related FAs eicosadienoic acid (C20:2\omega 6) and its elongation product docosadienoic acid (C22:2\omega 6) \cite{14}. In addition, also other 20-carbon FAs (C20, C20:2\omega 6, and C20:1\omega 9) showed a significant linear relation of increased concentrations with one or two A-alleles. Interestingly, previous research in healthy and CVD-subjects did not find consistent associations of the Ala54Thr-polymerorphism with FA-concentrations \cite{24, 26, 27}. This could be explained by lack of power \cite{24, 27}, use of other sample mediums \cite{24, 26, 27}, lack of measurement of eicosadienoic acid (C20:2\omega 6) and docosadienoic acid (C22:2\omega 6), or inclusion of different patient populations \cite{24, 26, 27}.

Regarding other FAs, two of these previous studies found no associations \cite{24, 26}, while one other study \cite{27} found lower palmitoleic acid (C16:1\omega 7) and higher \alpha-linolenic (C18:3\omega 3) and lignoceric acid (C24:0) in phospholipids to be associated with threonine coding \textit{FABP2}-alleles. Interestingly, a study investigating \textit{FABP2}'s effect on FA-uptake suggested that threonine coding \textit{FABP2}-alleles are associated with an increased uptake of particularly 14-18-carbon FAs, including \alpha-linoleic acid (C18:2\omega 6) \cite{21}, which can be enzymatically elongated to eicosadienoic acid (C20:2\omega 6) and docosadienoic acid (C22:2\omega 6) \cite{14}. It may therefore be hypothesized that the increased red blood cell membrane concentration of eicosadienoic acid in our patients with the AA-genotype may reflect an adaptive conversion of increasingly bioavailable \alpha-linoleic acid (C18:2\omega 6), to prevent accumulation of its other, and supposedly more bioactive (as main precursor of pro-inflammatory eicosanoids), conversion product arachidonic acid (ARA; C20:4\omega 6) \cite{14}. Of note in this respect, increased ARA has been associated with (visceral) obesity and CVD-risk \cite{36, 37}. 
Interpretation of increased eicosadienoic acid concentrations in AA-genotype patients as an adaptive process to prevent ARA-accumulation corresponds with the observed negative association of eicosadienoic acid and waist circumference. In addition, eicosadienoic acid mediated the lower waist circumference in AA-patients: the relation between FABP2 and waist circumference lost significance when eicosadienoic acid was incorporated in the model. This might imply that for AA-patients, conversion of increasingly bioavailable α-linoleic acid (C18:2ω6) into eicosadienoic acid (C20:2ω6) instead of ARA (C20:4ω6) helps in maintaining a low waist circumference thereby possibly reducing CVD-risk.

4.4 Association between FABP2 and waist circumference

Our finding of an interaction at trend level between patient-status and FABP2-genotype on waist circumference is intriguing. Contrary to our third hypothesis and results in controls, AA-genotype was significantly associated with a lower waist circumference in the MDD-patients. This may have several explanations. First, patients’ metabolic constitution may be influenced by other genes, e.g. mutations in the 1-carbon-cycle, interacting with FABP2’s effect on waist circumference 38. In addition, stress may affect FA-metabolism leading to different associations in patients, through the association between the HPA-axis and FA-metabolism 39. Finally, FABP2’s effects may interact with dietary availability of nutrients, which could differ between patient and controls 16. Future research should investigate these possibilities.

Interestingly, several studies also indicate that the Ala54Thr-polymorphism may be associated with a beneficial CVD-risk profile in response to certain dietary regimens, including eicosapentaenoic acid supplementation 40, or moderate-fat or high-polyunsaturated fat diets 41, 42. In these studies, Ala54Thr-polymorphism carriers had better metabolic responses and e.g. a larger reduction in waist circumference. This may indicate that different dietary preferences in patients played a role in the interaction between patient-status and FABP2 on waist circumference. Unfortunately, a main limitation of the present study is that no dietary data has been collected. Nevertheless, given the genetic nature of our observed effects, dietary intake likely is not a confounding factor, but rather interacts with FABP2-genotype to explain observed relations. In addition, erythrocyte FA-concentrations are thought to be more stable than plasma FAs, thereby reflecting long-term FA-metabolism, instead of dietary fluctuations 17. Future nutrigenetic studies combining FABP2 and dietary assessment will further elucidate their interaction in explaining altered FA-metabolism and waist circumference in MDD(-R).
4.5 Additional limitations
Some additional limitation should be mentioned. Analyses did not differentiate between FA-subclasses, e.g. sphingomyelin or phosphatidylcholine. More advanced lipidomic analyses may provide better insight into the impact of \textit{FABP2} on FA-metabolism. However, this is the first report including a wide range of different FAs and indices into the analyses. This brings up another important point: the issue of multiple testing. Because of the multitude of FAs, studying FA-metabolism usually entails multiple comparisons. Although often overlooked in FA-literature, multiple comparisons may lead to type-I errors. We dealt with this intrinsic problem by (I) applying pathophysiologically driven data reduction using the UI, CLI and PI, and (II) a priori designating several outcomes as hypothesis based, and others as explorative. However, despite these precautions, it remains important to keep the multiple testing problem in mind when interpreting results. None of the reported significant results would have survived Bonferroni correction. However, this correction likely would have been too strict considering the relatively strong assumed correlations between the different outcomes in this study. For that reason, strict correction in this sample could have induced type-II errors, and therefore we consequently chose to correct neither the results of the a priori hypothesized outcomes, nor the explorative results, for multiple comparisons. Therefore, particularly explorative first results should be interpreted as such, and replication in further investigations is warranted. In addition, while power calculations showed that we had adequate power to detect small effect sizes for almost all analyses, we had insufficient power to detect small effect sizes in the analyses on the differences between the different genotype classes in FA-measures and waist circumference in the patient group, for which we were able to detect medium effect sizes ($f > 0.268$). Therefore, we may have missed additional small effects of \textit{FABP2} on FA-measures or waist circumference. However, for these analyses, we additionally operationalized the effects of \textit{FABP2} as a linear function, which resulted in adequate power to detect small effect sizes ($f^2 > 0.080$). So, in conclusion, the only effects for which our set-up may have precluded detection of small effect sizes would be non-linear effects of \textit{FABP2} on FA-measures and/or waist circumference. However, both (I) existing literature on the physiological role of \textit{FABP2}, and (II) data from our sample presented in table 1, do not suggest such non-linear relationships.

4.6 Study strengths
Our study also has particular strengths. To our knowledge \textit{FABP2} was investigated in a psychiatric population for the first time, in a specific sample of MDD-R-patients with known bimodally distributed FA-alterations. In addition, an advanced multiple imputation procedure reduced the possibility that missing data have influenced results. Finally, this is the first
report of an association between FABP2 and two specific mutually biologically related FAs, namely eicosadienoic acid (C20:2ω6) and docosadienoic acid (C22:2ω6), which increases knowledge on FABP2’s role in health and disease.

5. Conclusion
Although Ala54Thr-polymorphism distribution was not associated with MDD, A-allele carrying patients had (I) higher concentrations of several 20-carbon FAs, particularly eicosadienoic acid (C20:2ω6), and associated (II) lower waist circumference. Therefore, for AA-patients, potentially adaptive conversion of increasingly bioavailable dietary precursors into eicosadienoic acid might mediate maintenance of a low waist circumference, which may guide future investigations of CVD-prevention in these patients. Considering the explorative nature of this first investigation of these associations, replication is warranted, preferably by nutrigenetic studies applying lipidomics and detailed dietary assessment.

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Chapter 5
One-carbon metabolism
One-carbon metabolism

5.1 The one-carbon-cycle and methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism in recurrent Major Depressive Disorder; influence of antidepressant use and depressive state?

Lok A, Mocking RJT, Assies J, Koeter MWJ, Bockting CLH, de Vries GJ, Visser I, Derks EM, Kayser M, Schene AH

submitted for publication
Abstract

Background
An important biological factor suggested in the pathophysiology of (recurrent) Major Depressive Disorder (MDD) concerns a polymorphism in a gene encoding for the MTHFR enzyme of the one-carbon (1-C)-metabolism. Integratively investigating key 1-C-components (folate, homocysteine, vitamin B₆ and B₁₂), including the possible effects of antidepressant medication and depressive state, could provide more insight in the possible association between the MTHFR-polymorphism and recurrent MDD.

Methods
We compared the MTHFR C677T-polymorphism together with the key 1-C-components in clinically ascertained patients with recurrent MDD (n=137) to age- and gender-matched healthy controls (n=73).

Results
First, patients had lower folate (t=2.25; p=.025) as compared to controls; a difference that resolved after correction for demographics (t=1.22; p=.223). Second, patients that were depressed during sampling had lower vitamin B₆ (t=-2.070; p=.038) and higher homocysteine (t=2.404; p=.016) compared to those in remission. Finally, current use of antidepressants had no influence on the 1-C-components.

Conclusions
Despite investigation of a specific recurrently depressed patient population, we found no clear associations with the 1-C-cycle, except for higher homocysteine and lower vitamin B₆ during the depressed state. This suggests that 1-C-cycle alterations in MDD are state-associated, possibly resulting from high levels of acute (psychological) stress, and may provide a treatment target to reduce cardiovascular risk in this population.

Clinical Trial registration
ISRCTN 68246470 http://www.controlled-trials.com/ISRCTN68246470/bockting
1 Introduction

Major depressive disorder (MDD) is a common psychiatric disorder with high morbidity, economic burden and rates of completed suicide \(^1,2,3\). Biological studies in MDD-patients, MDD-remitted patients, their family members and persons at MDD-risk showed that metabolic and hormonal disturbances observed in MDD are also present preceding, and subsequent to the depressed state or episode, suggesting heritable traits \(^4,5,6,7,8\). This may particularly hold true for the severe and recurrent form of MDD, considering its higher heritability than mild, moderate and/or single episode forms of MDD \(^9,10\). More knowledge on neurobiological and genetic factors of MDD may lead to (I) better understanding of MDD’s nature and course, and thereby to improved (II) prevention and (III) treatment \(^12,13,11\).

An important biological factor suggested in MDD concerns genetic variation in the folate metabolism [see figure 1] \(^14,15\). Folate functions as an essential coenzyme in one carbon transfers, which are crucial for methylation processes such as (I) metabolism of amino acids, phospholipids and neurotransmitters, and (II) DNA-synthesis, -repair and -methylation. A link between the folate-/methylation cycle [labelled as the one-carbon (1-C) cycle] and MDD seems biologically plausible \(^16\). 5-Methyltetrahydrofolate (5-MTHF), the pre-dominant circulating form of folate, donates a methyl group to homocysteine during generation of S-adenosylmethionine (SAMe), the major source of methyl groups in the brain. Therefore, genetically determined changes in the activity of 1-C-cycle enzymes like 5,10-methylenetetrahydrofolate reductase (MTHFR) may contribute to disturbances in neurocognitive functioning and mood regulation, predisposing to development and adverse course of MDD \(^17,18\).

The common single base 677C→T mutation of the gene encoding the enzyme MTHFR is associated with reduced activity and so with decreased methylation capacity \(^19\). This is associated with a pattern of significant elevation in circulating homocysteine and a decrease in serum folate concentrations \(^20\), which may parallel a similar 5-MTHF reduction in the central nervous system (CNS). Considering the role of these folic metabolic pathway-alterations in the brain, the 677C→T MTHFR-mutation could be one of the explanations for the heritable and recurrent nature of MDD \(^21,14,22,23\).

Two meta-analyses indicated that the MTHFR TT-homozygous genotype increases MDD-risk \(^24,25\). However, two other meta-analyses found no association between the MTHFR-gene variants and MDD \(^26,27\). A recent meta-analysis of all published case-control studies investigating MTHFR C677T in 9648 patient and 19,854 control subject showed that carriers of the T-allele
Figure 1

1-Carbon-Cycle
The metabolic relationship between the folate and the methylation cycle

Legend
The Methylation Pathway: All methylation reactions that utilize S-adenosylmethionine (SAM) as the methyl-donor generate S-adenosylhomocysteine (SAH). The methyl group from 5-methyltetrahydrofolate (MTHF) is transferred to Hcy to form methionine and tetrahydrofolate (THF). This de novo synthesis of methionine requires vitamin B12, which is directly involved in the transfer of the methyl group to Hcy.

The transulfuration pathway: involves condensation with serine to form cystathionine by the enzyme cystathionine-β-synthetase (CBS). In this reaction homocysteine is committed to the transulfuration pathway as it is converted to cystathionine, leading to the formation of glutathione (GSH), a major cellular antioxidant. Vitamin B6 (pyridoxal phosphate) is required as a cofactor for CBS activity.
and TT-genotype are at a small (odds ratio=1.26) but significant increased risk of major psychiatric disorders like schizophrenia, bipolar disorder and unipolar MDD. Recently we found a gene by environment interaction between the MTHFR-gene and traumatic childhood events on recurrence in MDD. T-allele carriers were at increased risk for future depressive symptoms after exposure to childhood trauma. So, thus far, data linking polymorphisms in the MTHFR-gene with MDD are still contradictory.

Another inconsistent pattern emerges when looking at the associations between MDD and four key constituents of the 1-C-cycle: homocysteine, folate, vitamin B6 and vitamin B12. Both high homocysteine and low folate have been linked with MDD. There is also evidence suggesting that vitamin B6 and vitamin B12 may be associated with MDD-symptomatology. Nevertheless, thus far, no consistent associations are found between these 1-C-cycle components and MDD-symptomatology.

Several methodological issues may explain these inconsistencies; such as heterogeneous populations, small sample sizes and non-uniformity in diagnostic measures (e.g. DSM-IV diagnosis versus severity of depressive symptoms). For example, the association of MTHFR-polymorphisms with MDD may be stronger for the recurrent and more heritable type of MDD, which might indicate that the MTHFR 677T-genotype is in particular related to recurrency. However, to our knowledge, the association of MTHFR with recurrent MDD in particular has not yet been investigated. Moreover, most genetic MTHFR-studies in patients with MDD did not measure all four 1-C-cycle key components simultaneously. It is also unclear whether 1-C component alterations are state-dependent or rather consistent throughout the course of (recurrent) MDD. Furthermore, the role of antidepressant (AD)-use is not accounted for in most studies examining the MTHFR-genotype in MDD, despite AD’s effect on folate metabolism in depression. For example, selective serotonin re-uptake inhibitors (SSRIs) may reduce oxidative stress effects (e.g. lipid peroxidation and superoxide dismutase activity) in depressive patients, which could possibly result in 1-C metabolism alterations.

Integratively investigating 1-C components (folate, homocysteine, vitamin B6 and B12), while including possible effects of AD-medication and depressive state, could provide more insight in the possible association between the MTHFR C677T-polymorphism and recurrent MDD. Therefore, we examined the MTHFR C677T-polymorphism together with 1-C-cycle components in patients with recurrent MDD (n=137) and compared these to age- and gender-matched healthy controls (n=73).
We hypothesized that: (I) *MTHFR* 677TT- and the 677CT-genotypes are present more frequently in recurrent MDD-patients than in healthy controls; (II) Recurrent MDD-patients have lower folate, vitamin B₆ and B₁₂ and higher homocysteine compared to controls; (III) T-carrying patients have more pronounced alterations; (IV) differences in metabolites are more clearly expressed in the depressive state compared to the remitted state; and finally, (V) patients using antidepressants have lower homocysteine and higher folate, vitamin B₆ and B₁₂ compared to patients not using antidepressants.

2 Methods

2.1 Study population

The current study was part of the DELTA-study, a randomized clinical trial investigating the effect of 8-week cognitive therapy on recurrence in euthymic patients with ≥2 previous major depressive episodes (MDEs) in the last five years. Among the exclusion criteria were current or previous mania or hypomania (bipolar disorder), any psychotic disorder (current or previous), alcohol or drug abuse and predominant anxiety disorder. Participants were recruited from psychiatric centers and through media announcement. Since neither type of aftercare, nor AD use, was an inclusion or exclusion criterion for the study, with respect to these characteristics, the DELTA-sample can be considered representative for patients suffering from recurrent depression. The background and methodology of the DELTA-study is described in more detail previously.

At 2-years follow up of the original trial, we invited patients to participate in the present biological sub-study. Although all patients were in remission at inclusion in the original trial, at 2-years they could be depressed (due to a MDD-recurrence) or in remission during blood sampling. We also recruited age- and gender-matched healthy control subjects, without a personal and/or family history of major depression, through a diversity of media approaches. All participants provided written informed consent prior to enrolment. The ethics committee of the Academic Medical Center of the University of Amsterdam approved the study.

2.2 Measurements

2.2.1 Blood samples and biochemical analysis

We collected blood samples at patient’s home; control subjects came to the hospital. Each subject had 20ml of blood taken by venipuncture in a non-fasting state. We determined serum folate and vitamin B₆ and B₁₂ using an immunoassay. We separated plasma homocysteine within 4h of collection and stored it at -80 °C until analysis of all samples. We determined
homocysteine with isocratic high-performance liquid chromatography (HLPC) electrospray tandem mass spectrometry (MS-MS). Intra- and interassay coefficients of variation, linear in range from 2 to 150 µmol/l, were within 3.6% and 4%, respectively.

We determined MTHFR C677T-genotype (rs1801133) by a polymerase chain reaction (PCR) and HinfI restriction enzyme digestion as described previously 41. In short, we isolated DNA from blood using a filter-based method (QIAamp DNA Mini Kit, Qiagen Ltd, United Kingdom). We designed PCR primers using Primer 3 (available at http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR primer sequence was GCCAGGTACCACAAAGGC and TGGGTGGAGG-GAGCTTATG, PEX primer sequence GAGAAGGTGTCTLCGGGAG. We used a Matrix Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) mass spectrometer from Bruker Daltonics. To increase reliability, we genotyped all samples in duplicate. Based on these duplicates, the genotyping error rate was 3.7% 29.

2.2.2 Ethnicity
To assess ethnicity, we asked participants for ancestry of their grandparents. We included participants with 4 grandparents of white European ancestry in the Caucasian sub-group 42. In case this procedure did not lead to a known ethnicity, e.g. due to unknown grandparents, we genetically ascertained ethnicity by typing 24 ancestry-informative autosomal SNPs and inferred continental ancestry from the genotypes using a supervised STRUCTURE approach where we used HGDP-CEPH samples as reference dataset 43. We performed and presented our analyses in the whole group, as well as in the Caucasian subgroup.

2.2.3 Depression characteristics
2.2.3.1 Relapse/recurrence
For the current study, depressive state was assessed with the Structured Clinical Interview for DSM-IV (SCID-I) 44 by trained SCID evaluators. These interviewers (psychologist/research assistants) were blind to treatment condition; subjects were instructed not to reveal treatment condition. All interviews were audio taped. Two independent experienced psychiatrists, blind to treatment condition, evaluated all occasions of participants meeting the DSM-IV criteria for MDD. In cases of disagreement, ratings of the psychiatrists were used. Kappa for inter-rater agreement between the interviewers and psychiatrist on categorization of a relapse/recurrence or no relapse/recurrence was .96, indicating high agreement.
2.2.3.2 Antidepressants
We assessed antidepressant (AD) use during sampling-time with the Trimbos/IMTA Self Report Questionnaire for Costs Associated with Psychiatric Illness (TiC-P)\textsuperscript{45}. Current antidepressant use was operationalized dichotomously (yes/no) during this assessment.

2.3 Statistical analysis
2.3.1 Data handling
To prevent bias possibly introduced by missing values we used multiple imputation, using the package Amelia II\textsuperscript{46}, as described previously\textsuperscript{4}. In the imputation procedure, we included the independent and dependent variables of our models, and other variables that correlated with these variables, e.g. other biological parameters. This resulted in five imputed datasets on which we performed subsequent analyses.

Vitamins and homocysteine showed normal distributions after log transformations, which were used in the analyses throughout, except for vitamin B\textsubscript{12}. The \textit{MTHFR} C677T polymorphism variable was dichotomized into “T-carriers” (T allele carriers: \textit{MTHFR} 677TT and 677CT) and “non-T-carriers” (677CC) groups.

2.3.2 Hardy-Weinberg Equilibrium
To test for deviation from Hardy-Weinberg Equilibrium (HWE) in controls, a pooled chi-square test\textsuperscript{47} was calculated with a calculating program, available on http://www.oeg.oege.org/software/hwe-mr-calc.html.

2.3.3 Subject characteristics
Patients’ and controls’ baseline characteristics were compared using \(\chi^2\) and student’s \(t\)-test statistics.

In subsequent analyses we adjusted for confounders using propensity scores. Propensity scores are a way to correct for multiple confounders, while retaining adequate power in the analysis\textsuperscript{48}. Propensity scores are calculated for each analysis separately. They reflect the predicted probability for a given case to belong to a certain group of interest (e.g. patient or control), calculated in a binary logistic model with the chosen confounders as predictors. We calculated a propensity score (PS1) for comparisons between patients and controls that corrects for demographic variables: educational level (low, middle, high), marital status, social economic status and smoking behaviour for both patients and controls\textsuperscript{14,30,49}. We created an additional
propensity score (PS2) to adjust effect estimates in the patient analyses, which, in addition to the confounders in PS1, also correct for the disease-related variables: age of MDD-onset, number of depressive episodes in the last 2 years, life events before the age of 16, HDRS score and current AD use.

2.3.4 Comparison of recurrent MDD-patients and controls on 1-carbon metabolite concentrations
Differences between the recurrent MDD-patient- and control group were assessed with linear regression analyses with 1-carbon metabolite concentration as dependent and “group” (recurrent MDD-patients or controls) as independent variable. In all analyses we corrected for potential confounding demographic variables available for both patients and controls using PS1. We did not correct for variables of which we suspect to be an intermediate step in the causal path (e.g. waist circumference) 50.

2.3.5 Effect of depressive state
To detect differences between currently depressed MDD-patients and non-depressed patients, we performed linear regression analyses with 1-carbon metabolite concentration as dependent and “state” (currently depressed MDD-patients or euthymic MDD-patients) as independent variable. In this comparison we corrected for PS1 and PS2.

2.3.6 Effect of antidepressant (AD) use
Differences between patients who are currently AD-users and those without AD-medication were assessed with linear regression analyses with 1-carbon metabolite concentration as dependent variable and “AD-use” (current AD use or non-AD use) as independent variable. In this comparison we corrected for PS1 and PS2.

2.3.7 Comparison of T-carriers and non-T carriers on metabolite concentrations in MDD-patients
For each metabolite, the effect of MTHFR-polymorphism on the concentration was assessed with linear regression analyses with metabolite concentration as dependent variable and MTHFR-polymorphism as predictor.

We used PASW statistics 18.0 (SPSS, Inc., 2009, Chicago, IL). For estimates that could not be pooled by SPSS, we combined separate significance tests for the five imputed datasets into one pooled test with an SPSS macro from Van Ginkel (2006) 51 following Rubin’s rules 52.
We considered \( p < .05 \) statistically significant. In line with previous research \cite{22,29} and because our tests were hypothesis based, we chose not to correct for multiple testing.

3 Results
Of the 172 patients of the original trial, 137 participated in the current study at 2-years follow-up. Of the 35 patients that were not assessed; 15 (8.7\%) were lost to follow-up, and the remaining patients (11.6\%) did not participate due to diverse reasons (e.g. being afraid of needles, ethical issues regarding genetic studies). In addition, seventy-three controls were included in this study. Of the 137 included patients, one reported non-Caucasian ethnicity and 6 had missing ethnicity. Of the 73 included controls, one reported non-Caucasian ethnicity and 21 had missing ethnicity. Genetic ancestry analyses showed that all missing patient ethnicities were Caucasian, while of the missing controls ethnicities two individuals showed admixture between Western Eurasia and E-Asia/Native America, and one seemed more admixed with a stronger E-Asian component but also showing admixture from other regions. C677T-polymorphism genotype frequency distribution was in Hardy-Weinberg equilibrium (HWE) in controls \( F(1,4218) = .049, \ p = .48 \).

Of the 137 patients who responded and provided a blood sample, no values could be obtained for respectively: folate \( n = 1 \); vitamin \( B_6 \) \( n = 4 \); vitamin \( B_{12} \) \( n = 1 \), homocysteine \( n = 5 \) and \( MTHFR \)-genotyping \( n = 5 \). Of the 73 controls, no values could be obtained for respectively; folate \( n = 6 \), vitamin \( B_6 \) \( n = 3 \), vitamin \( B_{12} \) \( n = 4 \), homocysteine \( n = 10 \) and \( MTHFR \)-genotyping analyses \( n = 3 \). These missing values were all due to technical reasons. Standard diagnostics of Amelia II suggested successful multiple imputation.

3.1 Participant characteristics
Demographics and psychopathological characteristics are depicted in table 1. As expected, there were no statistically significant differences in gender or age between patients and controls. Most patients were currently recovered (81\%) from depression while one in five was depressed at the time of sampling. Selective serotonin reuptake inhibitors (SSRIs) were the most commonly described ADs (64 \%).
Table 1: Subject Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n = 137)</th>
<th>Controls (n = 73)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>36/101</td>
<td>22/51</td>
<td>.551</td>
</tr>
<tr>
<td>Age, mean (SD), year</td>
<td>46.4 (9.5)</td>
<td>44.7 (9.4)</td>
<td>.205</td>
</tr>
<tr>
<td>Educational level&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>- Low, %</td>
<td>33.3</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>- Middle, %</td>
<td>31.2</td>
<td>22.5</td>
<td></td>
</tr>
<tr>
<td>- High, %</td>
<td>35.5</td>
<td>72.3</td>
<td></td>
</tr>
<tr>
<td>Single, %</td>
<td>38.4</td>
<td>30.1</td>
<td>.265</td>
</tr>
<tr>
<td>Social class&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>- Class 1, %</td>
<td>55.0</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>- Class 2, %</td>
<td>32.0</td>
<td>52.1</td>
<td></td>
</tr>
<tr>
<td>- Class 3, %</td>
<td>13.0</td>
<td>36.7</td>
<td></td>
</tr>
<tr>
<td>Smoking, %</td>
<td>39.4</td>
<td>49.0</td>
<td>.211</td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>26.0 (4.8)</td>
<td>24.8 (3.5)</td>
<td>.062</td>
</tr>
<tr>
<td>Waist circumference, mean (SD), cm</td>
<td>89.3 (13.9)</td>
<td>84.9 (12.1)</td>
<td>.025</td>
</tr>
<tr>
<td>In Caucasian subset, %</td>
<td>99.3</td>
<td>91.8</td>
<td>.047</td>
</tr>
<tr>
<td>AD-use during sampling, %</td>
<td>62.8</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>- of which SSRIs, %</td>
<td>64.0</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Received cognitive therapy, %</td>
<td>54</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>HDRS17 score, mean (SD)</td>
<td>5.9</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Number of previous episodes, mean (SD)</td>
<td>7.7</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Depressed during sampling, %</td>
<td>19</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Age of first onset, years</td>
<td>28.4</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Psychiatric diseases first relatives (%)</td>
<td>68</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations** - AD = antidepressant (missing n=5); HDRS = Hamilton depression rating scale; SSRI = Selective Serotonin Reuptake Inhibitor.

<sup>a</sup> Educational level is defined as: low, primary education or preparatory middle-level applied education; middle, higher general continued education or middle-level applied education; and high, preparatory scientific education, higher applied education or scientific education.

<sup>b</sup> Class 1, e.g. cleaner; Class 2, e.g. nurse; Class 3, e.g. general manager. Group comparisons were calculated using students-t tests, $\chi^2$ test of Fisher exact test.

3.2 *MTHFR*-genotype in patients and controls
Frequency of the T-carrying genotype did not differ between patients and controls (p=.856). Triallelic distributions also showed no significant (p=.576) differences: 677TT-genotype
(patients 12.3% vs. controls 8.5%), 677CT-genotype (patients 41.2% vs. controls 47.4%) and 677CC (patients 46.6% vs. controls 44.1%). The MTHFR 677TT was 45% more prevalent in the MDD-patients than in controls.

3.3 1-C components in patients and controls
Homocysteine, vitamin B₆ and B₁₂ were not statistically different between the recurrent MDD-patients and their controls (table 2). Patients had significantly lower folate compared to controls ($t=2.25$, $p=.025$). This difference was no longer significant after adjustment for demographic confounders ($t=1.22$, $p=.223$).

Table 2: Plasma concentrations of metabolites of the one-carbon cycle: patients with recurrent depression ($n=137$) and controls ($n=73$)

<table>
<thead>
<tr>
<th></th>
<th>Uncorrected</th>
<th>Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>Folate ± SE</td>
<td>3.12 ± 0.03</td>
<td>3.23 ± 0.04</td>
</tr>
<tr>
<td>Vitamin B₆ ± SE</td>
<td>4.37 ± 0.05</td>
<td>4.49 ± 0.07</td>
</tr>
<tr>
<td>Vitamin B₁₂ ± SE</td>
<td>314.1 ± 10.6</td>
<td>304.0 ± 14.7</td>
</tr>
<tr>
<td>Homocysteine ± SE</td>
<td>2.23 ± 0.03</td>
<td>2.30 ± 0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Corrected</th>
<th>Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>Folate ± SE</td>
<td>3.14 ± 0.03</td>
<td>3.20 ± 0.04</td>
</tr>
<tr>
<td>Vitamin B₆ ± SE</td>
<td>4.37 ± 0.05</td>
<td>4.49 ± 0.08</td>
</tr>
<tr>
<td>Vitamin B₁₂ ± SE</td>
<td>310.3 ± 11.2</td>
<td>311.0 ± 16.0</td>
</tr>
<tr>
<td>Homocysteine ± SE</td>
<td>2.23 ± 0.03</td>
<td>2.30 ± 0.04</td>
</tr>
</tbody>
</table>

* Corrected for educational level, social economic status, smoking and marital status.
* Log transformed.
3.4 Relation between 677T-carrying genotype and 1-C components
Homocysteine, vitamin B₆ and B₁₂ concentrations were not statistically different between T-carrying and non-T-carrying patients (see Table 3). Folate concentrations of 677T-carrying patients were 3.070 ± 0.04; in non-T-carrying patients 3.168 ± 0.04 (t=1.77, p=.078).

Table 3: Plasma concentrations of metabolites of the one-carbon cycle: patients with recurrent depression with a T-carrying 5,10-methylenetetrahydrofolate reductase (MTHFR) genotype (n = 71) vs. a non T-carrying MTHFR genotype (n = 66)

<table>
<thead>
<tr>
<th></th>
<th>T-carrying</th>
<th>Non-T-carrying</th>
<th>t</th>
<th>P</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folatea ± SE</td>
<td>3.070 ± 0.04</td>
<td>3.168 ± 0.04</td>
<td>-1.766</td>
<td>.078</td>
<td>-1.801</td>
<td>.072</td>
</tr>
<tr>
<td>Vitamin B₆a ± SE</td>
<td>4.364 ± 0.07</td>
<td>4.353 ± 0.07</td>
<td>-0.110</td>
<td>.912</td>
<td>-0.204</td>
<td>.839</td>
</tr>
<tr>
<td>Vitamin B₁₂ ± SE</td>
<td>306.5 ± 15.2</td>
<td>321.8 ± 15.8</td>
<td>-0.694</td>
<td>.488</td>
<td>-0.746</td>
<td>.456</td>
</tr>
<tr>
<td>Homocysteinea ± SE</td>
<td>2.267 ± 0.04</td>
<td>2.196 ± 0.04</td>
<td>1.346</td>
<td>.179</td>
<td>1.396</td>
<td>.163</td>
</tr>
</tbody>
</table>

a Log transformed.

3.5 Depressive state and 1-C components
Patients being in a Major Depressive Episode during blood sampling had lower vitamin B₆ and higher homocysteine concentrations, compared to patients in remission (table 4). This difference remained significant after correction for demographic factors, also in the Caucasian subset. Additional correction for clinical variables resolved significance, although differences remained relatively similar - particularly for vitamin B₆.
Table 4: Plasma concentrations of metabolites of the one-carbon cycle: currently depressed patients \((n = 26)\) vs. remitted patients \((n = 111)\); uncorrected and corrected for confounders

<table>
<thead>
<tr>
<th></th>
<th>Caucasian subset</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncorrected</td>
<td>Corrected(^a)</td>
<td>Corrected(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Depressed</td>
<td>Remitted</td>
<td>(t)</td>
<td>(P)</td>
<td>(t)</td>
<td>(P)</td>
</tr>
<tr>
<td>Folate(^c)  ± SE</td>
<td>3.048 ± 0.06</td>
<td>3.134 ± 0.03</td>
<td>-1.215</td>
<td>.224</td>
<td>-1.228</td>
<td>.219</td>
</tr>
<tr>
<td>Vitamin B(_6) ± SE</td>
<td>4.141 ± 0.12</td>
<td>4.409 ± 0.06</td>
<td>-2.070</td>
<td>.038</td>
<td>-2.118</td>
<td>.034</td>
</tr>
<tr>
<td>Vitamin B(_12) ± SE</td>
<td>304.8 ± 25.0</td>
<td>315.9 ± 12.1</td>
<td>-0.399</td>
<td>.690</td>
<td>-0.423</td>
<td>.672</td>
</tr>
<tr>
<td>Homocysteine(^c) ± SE</td>
<td>2.359 ± 0.06</td>
<td>2.203 ± 0.03</td>
<td>2.404</td>
<td>.016</td>
<td>2.425</td>
<td>.015</td>
</tr>
</tbody>
</table>

\(^a\) Corrected for age, sex, educational level, social economic status, smoking behaviour and marital status.

\(^b\) Corrected for age, sex, educational level, social economic status, smoking behaviour and marital status, age of MDD-onset, number of episodes last 2 years, life events before the age of 16, and current AD use.

\(^c\) Log transformed.
3.6 Influence of antidepressant use

Current antidepressant use was not associated with 1-C cycle alterations ($p$'s all $>.157$; supplementary table).

Supplementary table: Plasma concentrations of metabolites of the one-carbon cycle: patients with recurrent depression currently using antidepressants (AD) ($n = 51$) vs currently not using AD ($n = 86$); uncorrected and corrected for confounders and HDRS-scores

<table>
<thead>
<tr>
<th></th>
<th>Uncorrected</th>
<th>Caucasian subset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AD</td>
<td>No AD</td>
</tr>
<tr>
<td>Folate $^c \pm SE$</td>
<td>3.084 ± 0.03</td>
<td>3.174 ± 0.04</td>
</tr>
<tr>
<td>Vitamin B$_6$ $^c \pm SE$</td>
<td>4.316 ± 0.06</td>
<td>4.430 ± 0.08</td>
</tr>
<tr>
<td>Vitamin B$_{12}$ $^c \pm SE$</td>
<td>310.7 ± 13.8</td>
<td>319.0 ± 17.8</td>
</tr>
<tr>
<td>Homocysteine $^c \pm SE$</td>
<td>2.263 ± 0.03</td>
<td>2.181 ± 0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Corrected $^a$</th>
<th>Caucasian subset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AD</td>
<td>No AD</td>
</tr>
<tr>
<td>Folate $^c \pm SE$</td>
<td>3.088 ± 0.04</td>
<td>3.168 ± 0.05</td>
</tr>
<tr>
<td>Vitamin B$_6$ $^c \pm SE$</td>
<td>4.323 ± 0.07</td>
<td>4.417 ± 0.09</td>
</tr>
<tr>
<td>Vitamin B$_{12}$ $^c \pm SE$</td>
<td>309.2 ± 14.0</td>
<td>321.6 ± 18.5</td>
</tr>
<tr>
<td>Homocysteine $^c \pm SE$</td>
<td>2.254 ± 0.03</td>
<td>2.197 ± 0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Corrected $^b$</th>
<th>Caucasian subset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AD</td>
<td>No AD</td>
</tr>
<tr>
<td>Folate $^c \pm SE$</td>
<td>3.086 ± 0.04</td>
<td>3.170 ± 0.05</td>
</tr>
<tr>
<td>Vitamin B$_6$ $^c \pm SE$</td>
<td>4.326 ± 0.07</td>
<td>4.412 ± 0.09</td>
</tr>
</tbody>
</table>

*Table is continued on the next page*
Time after Time: biological factors in the course of recurrent depression

<table>
<thead>
<tr>
<th>Vitamin B₁₂ ± SE</th>
<th>309.8 ± 14.1</th>
<th>320.6 ± 18.6</th>
<th>-0.454</th>
<th>.650</th>
<th>-0.534</th>
<th>.594</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine² ± SE</td>
<td>2.252 ± 0.03</td>
<td>2.201 ± 0.04</td>
<td>0.923</td>
<td>.356</td>
<td>0.991</td>
<td>.322</td>
</tr>
</tbody>
</table>

* Corrected for age, sex, educational level, social economic status, smoking behaviour and marital status
* Corrected for age, sex, educational level, social economic status, smoking behaviour and marital status, age of MDD-onset, number of episodes last 2 years, life events before the age of 16, HDRS score
* Log transformed.

4 Discussion

We studied the MTHFR gene and four key components of the one-carbon-cycle (folate, homocysteine, vitamin B₆ and B₁₂) in a sample of patients with recurrent MDD, and made a comparison with controls. Our hypotheses were only partially confirmed. We found that the genotype distribution did not significantly differ between both groups, although the MTHFR 677TT-genotype was 45% more prevalent in MDD-patients than in controls. We found no differences in vitamin B₆, vitamin B₁₂ and homocysteine levels between T-carrying vs non-T-carrying MDD patients. We observed lower folate in patients compared to controls, but this difference dissolved after correction for demographics. Furthermore, results partially confirmed our hypothesis of an influence of depressive state, in that patients who were depressed during sampling we found lower vitamin B₆ and higher homocysteine compared to those in remission. Finally, current AD-use was not associated with 1-C-cycle alterations.

So, even in this specific, severely affected population of MDD-R patients, results were in line with earlier reports that showed no consistent abnormalities in MTHFR-genotype and 1-C-cycle metabolism. Nevertheless, some 1-C-cycle involvement in certain MDD-R aspects cannot be ruled out. For example, the 45% increased TT-genotype prevalence suggests that in interaction with other genes and/or environmental factors it might increase MDD-R vulnerability 41. However, both biallelic and triallelic genotype distributions were not significantly different between patients and controls. This may be due to relatively small numbers carrying the TT-genotype. Meta-analysis of future studies also in specific very high-risk clinical samples is needed to rule out the possibility that MTHFR is involved in MDD-R.

Of interest, folate concentrations of 677T-carrying patients were lower than in non-T-carrying patients. While this difference was not significant ($p= .078$), the direction was in line with our hypothesis. Cohen’s d for this effect was 0.304, indicating that possibly a small effect has failed to reach significance due to relatively low power, inherent to research in such a specific clinical
sample. This would be in accordance with a previous study, which showed a trend of an effect of T-allele on lower folate. Interestingly, low folate has been associated with MDD-severity and prolonged MDD-episodes. However, on the basis of our data and previous research, we refrain from drawing firm conclusions.

With regard to our second hypothesis, results showed lower concentrations of folate in patients compared to controls. The fact that this effect vanished after correction for demographic confounders implies it is mainly caused by environmental factors, e.g. diet. However, lower observed folate in patients may remain important in explaining increased cardiovascular risk. We observed no differences in the other 1-C-cycle metabolites between patients and controls. Overall, previous studies also showed considerable variation in strength and degree of associations between folate, homocysteine, B6 and B12 vitamins and MDD and depressive symptoms. Clinical studies linked folate deficiency, low folate status, or high homocysteine to MDD with persistent depressive symptoms, especially in elderly people. In the largest sample examined to date concerning psychiatric symptomatology, a cross-sectional study of 11,757 participants, a significant positive relationship was found between elevated homocysteine and current depressive symptoms; elevated homocysteine was associated with 26% greater odds of currently experiencing depressive symptoms. However, Penninx et al. found no association between folate and homocysteine and MDD. In addition, Morris et al. observed that homocysteine was not associated with MDD-diagnosis in young populations. Some of the reported variation in these associations may be attributed to geographical, dietary habits, and genetic differences between various populations. However, data from our fourth hypothesis suggest clinical characteristics may play a role as well.

Because notably, this hypothesis was partially confirmed by the finding that patients who were depressed during sampling had higher homocysteine and lower vitamin B6 compared to those in remission. Differences remained after correction for demographic confounders, and although statistical significance was lost after correction for clinical characteristics (p=.051), differences remained similar. This suggests that significance loss was mainly due to reduced power as a result of included confounders (despite use of propensity scores to correct for multiple confounders while retaining power). This difference in homocysteine and particularly vitamin B6 between patients who were depressed during sampling as compared to those who were in remission, may imply that 1-C-cycle metabolites alterations represent a state, only present during a high level of experienced depressive symptoms. Depressive state, and accompanying (psychological) stress, could adversely influence life-style (e.g. dietary intake) and
thereby result in lower circulating vitamin \( B_6 \) and higher homocysteine. Importantly, vitamin \( B_6 \) has pathophysiological roles that could underlie a relation with MDD in that (I) its primary biologic form, pyridoxal 5'-phosphate, is a cofactor in neurotransmitters synthesis, including serotonin, and (II) it was inversely associated with systemic markers of inflammation and oxidative stress independently of intake and plasma homocysteine \(^{56,57}\).

With regard to the effect of AD-use, results were contradictory to our fifth hypothesis: observed trends of lower folate and higher homocysteine in AD-users, which disappeared after correction. This may suggest that observed trends were caused by confounding effects due to the fact that patients who need AD are generally more ill than those who do not use ADs. This implies that possible protective AD-effects, although unlikely and presumably small, could have been masked by AD-related clinical confounders. Comparably, AD-use in patients may have distorted differences in 1-C-cycle components between patients and controls. Future RCT’s could provide more clarity about AD-influence on the 1-C-cycle in recurrent MDD.

Interestingly, overall, differences found in this study are smaller than expected on the basis that we compared high-risk recurrence MDD-patients with controls, where we hypothesized the greatest contrast would be because recurrent MDD is a more heritable and so presumably more biologically determined type of MDD. This could mean that differences in previous studies may relate to MDD in general and are not specific related to more severe MDD-types.

The small 1-C-cycle alterations, mostly disappearing after correction, were found to be state-dependent. So rather than being the cause, the alterations may probably be consequence of the disease. This would be in accordance with earlier studies suggesting that the relationship between e.g. folate and MDD is state-dependent \(^{16}\). This would imply associated alterations in appetite, food intake and overall lifestyle. Moreover, this could be a possible provocative mechanism whereby (psychological) stress during the depressive state may contribute to the initiation and/or progression of a more deleterious MDD-course and increased cardiovascular risk.

Our study has several limitations including the one-time sampling of the 1-C components. Also, data on diet, vitamin and other supplementations were not available at sampling time. Furthermore, our samples were neither drawn in the fasting state nor at a specific daily time. Another limitation is the possibility of selection bias because the final sample contained 137 patients of the 172 patients from the original trial. We lost a small part to follow up and the rest to a diversity of reasons (e.g. genotyping failure, being afraid of needles, ethical issues concerning genetic
study). However, patients of the final sample did not differ on the main clinical variables from those excluded from the analyses. This reduces the likeliness that this selection of 137 patients out of the original sample induced any additional selection bias.

Finally, although we used multiple imputation to reduce bias introduced by missing values, it could still have been that missing values influenced our results. However, most missing data in our study is missing completely at random, e.g. due to laboratory or logistic accidents, which in any case would not result in biases \(^58\). Furthermore, bias by missing values would not easily explain the observed results. However, replication of our findings would strengthen the evidence.

In spite of these limitations, our study is unique at providing the opportunity to investigate the role of the 1-C-cycle including the \textit{MTHFR}-genotype, in a well described and diagnostically assessed clinical sample of recurrent MDD-patients. In addition, these patients can be considered characteristic for those patients particularly causing the large MDD-associated burden of disease and of clinical relevance. More specific, seen as representatives of a more biologically determined MDD-subtype, findings in these patients may be specifically linked to recurrence and CVD-risk.

In sum, although the role of \textit{MTHFR}-genotype and 1-C-components was not clearly supported in our study with a high-risk population, we observed that higher homocysteine and lower vitamin \textit{B}_6 was associated with depressive state. Acute (psychological and associated oxidative) stress may induce a (transient) increase in homocysteine \(^59\) and decline of vitamin \textit{B}_6. Whatever the reason of altered 1-C-concentrations, e.g. a shared (patho)physiological mechanism combined with changed lifestyle, they are associated with a worse course of recurrent MDD. Our study does not permit causal inferences and the relationship between the 1-C-cycle components and affective state has to be prospectively further investigated in the course of MDD. Nevertheless, interference with 1-C-cycle components modifying or correcting the alterations may counterbalance negative effects in the recurrent course of MDD, e.g. in sub-groups of patients. Moreover, we should study and thereby differentiate influences of environment, genetics and stress on the 1-C-cycle.
Time after Time; biological factors in the course of recurrent depression

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One-carbon metabolism

5.2 Interaction between the \textit{MTHFR C677T} polymorphism and traumatic childhood events predicts depression

\textit{Lok A, Bockting CLH, Koeter MWJ, Snieder H, Assies J, Mocking RJT, Vinkers CH, Kahn RS, Boks MP, Schene AH}

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Abstract
Childhood trauma is associated with the onset and recurrence of Major Depressive Disorder (MDD). The thermolabile T variant of the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism (rs1801133) is associated with a limited (oxidative) stress defense. Therefore, C677T MTHFR could be a potential predictor for depressive symptomatology and MDD recurrence in the context of traumatic stress during early life.

We investigated the interaction between the C677T MTHFR variant and exposure to traumatic childhood events (TCEs) on MDD recurrence during a 5.5-year follow-up in a discovery sample of 124 patients with recurrent MDD and, in an independent replication sample, on depressive symptomatology in 665 healthy individuals from the general population.

In the discovery sample, cox regression analysis revealed a significant interaction between MTHFR genotype and TCEs on MDD recurrence (P=.017). Over the 5.5-year follow-up period, median time to recurrence was 191 days for T-allele carrying patients who experienced TCEs (T+ and TCEs+); 461 days for T- and TCEs+ patients; 773 days for T+ and TCEs- patients and 866 days for T- and TCEs- patients. In the replication sample, a significant interaction was present between the MTHFR genotype and TCEs on depressive symptomatology (P=.002).

Our results show that the effects of TCEs on the prospectively assessed recurrence of MDD and self-reported depressive symptoms in the general population depend on the MTHFR genotype. In conclusion, T-allele carriers may be at increased risk for depressive symptoms or MDD recurrence after exposure to childhood trauma.

Clinical Trial registration:
ISRCTN 68246470 http://www.controlled-trials.com/ISRCTN68246470/bockting
Introduction
Childhood maltreatment is associated with substantial and long-lasting cognitive and biological effects on the brain including heightened stress sensitivity. Therefore, individuals who have been exposed to childhood maltreatment are predisposed to an unfavorable course of major depressive disorder (MDD) and treatment outcome, as indicated by a recent meta-analysis. However, not all individuals exposed to traumatic stress develop a depression. Therefore, it is important to characterize the gene-environment interplay underlying the effects of traumatic childhood events (TCEs) on depression outcomes.

After a first episode of MDD, ~50% of patients will experience recurrences, which are responsible for considerable disability and impairment. Burcusa and Iacono stated as an explanation for recurrence in MDD that ‘individuals at high risk for multiple episodes possess the necessary characteristics to make them prone to recurrent depression, and such characteristics exist even before their first episode’. The recurrent type of MDD has a higher heritability than a single episode of MDD. Furthermore, biological studies in individuals at risk for MDD, or remitted from MDD, as well as their nondepressed family members, showed that pathophysiological disturbances also precede the development of MDD and remain present after remission, suggestive of stable heritable vulnerability traits, that is, endophenotypes. However, a direct identification of candidate genes with recurrence of MDD has proven to be difficult, presumably as a result of complex interactions between genes and environmental stressors.

A recently emerged pathway potentially underlying susceptibility to onset, symptomatology and recurrence of MDD is folate-mediated one-carbon (1-C) metabolism. The 1-C-cycle plays a central role in (1) the regulation of oxidative stress and (2) the generation of methyl groups for methylation of DNA, proteins, phospholipids and neurotransmitters. A crucial enzyme in this pathway is 5,10-methylenetetrahydrofolate reductase (MTHFR). A single-nucleotide polymorphism (SNP) in the MTHFR gene (C677T or rs1801133) results in the production of a thermolabile variant of MTHFR, which is associated with decreased methylation capacity and increased oxidative stress. This genetically determined variation in 1-C cycle activity associated with increased stress sensitivity may contribute to alterations in neurocognitive functioning and mood regulation.

Nevertheless, results of meta-analyses on data linking polymorphisms in the MTHFR gene with MDD have been inconsistent. From these studies it has become apparent that the main genetic effects overall are weak in MDD, whereas gene-environment interactions may...
provide stronger predictors\textsuperscript{27}. Investigating genetic susceptibility to stress is of particular relevance in the context of MDD as stress is considered one of the main pathogenic factors involved in depressive symptomatology and MDD recurrence: TCEs, recent life events, daily hassles and stress related to previous depressive episodes all pose increased risks for MDD and its recurrences\textsuperscript{1, 28, 29, 30, 31, 32}. Especially during early life, traumatic stress may result in lifelong programming, potentially through methylation-mediated alterations in expression of genes implicated in MDD\textsuperscript{33, 34, 35, 36, 37, 38, 39, 27}. As mentioned above, the thermolabile T variant of the MTHFR C677T polymorphism is associated with increased vulnerability to oxidative stress and a decreased DNA methylation capacity. Therefore, carriers of the MTHFR C677T variant may be particularly vulnerable to long-lasting effects of childhood traumatic stress on depressive symptomatology and MDD.

To study this proposed relation, we investigated the possible gene × environment interaction between TCEs and the MTHFR genotype as a potential predictor for depressive symptoms and recurrence in patients with a high risk of recurrence of depression over a 5.5-year follow-up period. We hypothesized that recurrently depressed patients carrying the T-allele would have a shorter time to MDD recurrence after exposure to TCEs, whereas this association would not be present in T-allele carriers without exposure to TCEs. Because the effects of the MTHFR genotype may not only be limited to MDD but also be present in the general population, we examined this gene-environment interaction in an independent population-based sample for replication.

Methods
Study participants
The current study was part of the DELTA study, a randomized clinical trial, investigating the effect of cognitive therapy on recurrence in 172 euthymic patients\textsuperscript{40, 41}. In the DELTA study, we sampled a group of patients with recurrent MDD. We considered these patients to suffer from a more biologically pronounced and endogenously determined subtype of MDD with a relatively high recurrence rate\textsuperscript{12}. Inclusion criteria of the original trial were: ≥2 previous MDD episodes in the past 5 years, as defined by the Structured Clinical Interview for DSM-IV disorders (SCID)\textsuperscript{42}; in remission >10 weeks and <2 years ago, as defined as a score <10 on the 17-item Hamilton Depression Rating Scale (HDRS\textsubscript{17})\textsuperscript{43}; and 18-65 years old. Exclusion criteria were: (a history of) bipolar spectrum disorder or any psychotic disorder, organic brain damage, alcohol and/or drug abuse and/or dependency or predominant anxiety disorder, all assessed by the SCID. The background, methodology and procedure of the DELTA study have been described in more detail previously\textsuperscript{40}. At 2 years after baseline, we asked the patients to provide DNA for the current
study. After complete description of the study to the subjects, written informed consent was obtained before enrolment. The study was approved by the ethics committee of the Academic Medical Center of the University of Amsterdam (MEC 02/048).

CannabisQuest cohort
Participants in the discovery sample were recruited using a project website launched in 2006 targeted at Dutch young adults and adolescents from age 18 to 25 years (www.cannabisquest.nl). Strategies to generate traffic on the project website included collaboration with over a hundred colleges, universities and youth centers, as well as the use of online commercial advertisement products (that is, banners and text links). The chance to win an Apple iPod or a Nintendo Wii was used as an incentive. Double entries were prevented by exclusion of subjects with an identical e-mail address, surname and date of birth. Anonymous submission of data was not possible. The online assessment included verification questions to protect against random answers, and participants failing to correctly complete the verification questions were subsequently excluded. From the online data (N=17,698), 1,259 participants were included for subsequent genetic assessment in two waves. First, in order to increase power for gene × environment interactions, in the context of cannabis and psychosis, we prioritized a sample of 719 participants who belonged to the top or bottom quintile of total scores of psychotic experiences as measured by the Community Assessment of Psychic Experiences (CAPE) score who were either cannabis naive (that is, a lifetime cannabis exposure frequency of <6 times) or were heavy cannabis users (that is, current expenditure for personal cannabis use exceeded 3 euro weekly). Second, an unselected sample of 540 individuals was included. As ascertained with the validated Dutch version of either the SCID or the MINI International Neuropsychiatric Interview, healthy controls had no history of any psychotic disorder. For 84 participants, no interview data were available, and for these cases the presence of a psychotic disorder was excluded by the absence of antipsychotic drug use or a history of psychiatric treatment. The possible concomitant use of recreational drugs was assessed with the substance abuse module of the Composite International Diagnostic Interview. Of the 1,259 participants who completed comprehensive assessments and provided blood samples for genetic testing, complete data were available for 665 subjects because of a later implementation of the Childhood Trauma Questionnaire (CTQ) assessment in the study. All participants provided a urine sample to screen for the presence of recreational drugs in order to verify recent self-reported cannabis use. The study was approved by the ethical review board of the University Medical Center Utrecht and all participants gave written informed consent.
Measurements
Depression

Discovery sample
Using SCID-I, current and past depressive episodes were assessed at baseline, and at 5 follow-up measurements at 3, 12, 24, 36 and 66 months after baseline. With these follow-up assessments, we diagnosed relapses (<6 months after a previous major depressive episode) or recurrences during follow-up, both further addressed as 'recurrence' for clarity reasons. The trained SCID evaluators were blind to treatment condition and subjects were instructed not to reveal their treatment condition to the interviewers (psychologist/research assistants). All interviews were audio taped. Two independent experienced psychiatrists, also blinded to treatment condition, evaluated all occasions of participants meeting the DSM-IV criteria for MDD. In cases of disagreement, we used the ratings of the psychiatrists. The \( \kappa \)-value for inter-rater agreement between the interviewers and psychiatrist on categorization of a relapse/recurrence or no relapse/recurrence was 0.96, indicating high agreement.

Replication sample
In the replication sample, the Beck Depression Inventory (BDI) was used to assess depressive symptoms. This validated 21-item self-report questionnaire measures current depressive symptoms during the last week. Each question has four possible answer choices ranging in intensity (0-3), resulting in a total BDI score ranging from 0 to 63.

Traumatic childhood events

Discovery sample
We defined TCEs as having experienced one of the following traumatic events before age 16: parental loss, sustained alcohol and/or drug abuse by caregiver, victim of a serious crime, victim of a serious accident and victim of sexual and/or physical abuse. We selected traumatic stress variables and stressors occurring to the self within this age period using the 7 items from the Negative Life Events Questionnaire that indicate traumatic events: items 5 and 8-13. This questionnaire proved to have a good predictive validity, as the number of negative life events predicted MDD symptom severity. We dichotomized the absence or presence of experienced TCEs.

Replication sample
In the replication sample, childhood maltreatment was assessed using the 25-item version of the CTQ. The CTQ assesses five types of self-report childhood trauma: emotional abuse, physical abuse, sexual abuse, emotional neglect and physical neglect. The validity of the CTQ,
including a Dutch translation, has been demonstrated in clinical and community samples \textsuperscript{48,51}. One translated item ("I believe I was molested") was excluded as this translation was found to be an invalid indicator of childhood sexual abuse in a previous validation study \textsuperscript{51}. Childhood maltreatment was used as the continuous sum score divided by the number of completed items. One item of the CTQ was only available for a subset of the replication sample ("My family was a source of strength and support"). Additional analyses in which this item was excluded altogether did not affect the results.

Genotyping procedures and analysis

\textit{Discovery sample}

We collected 20 ml blood samples at patients’ homes by venipuncture. DNA was isolated from blood using a filter-based method (QIAamp DNA Mini Kit, Qiagen, Manchester, UK). The PCR primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/input.htm). The PCR primer sequence was 5’-GGCAGGTTACCCAAAGGC-3’ and 5’-TGGGGTGGAGGGAGCTTATG-3’, and the PEX primer sequence was 5’-GAGAAGGTGTCTLCGGGAG-3’. Genotyping was done using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer from Bruker Daltonics (Wormer, The Netherlands). All samples were genotyped in duplicate to increase reliability \textsuperscript{52}. Genotyping error rate based on these duplicates was 3.7%.

\textit{Replication sample}

All participants were of Dutch ancestry. Genotype data were generated on three different array platforms: the IlluminaHumanOmniExpress (N=576), the IlluminaHuman610-QuadChip (N=768), and the IlluminaHumanHap550 array (N=34). For each SNP platform, quality control procedures were initially performed separately using PLINK V1.07.53 Subjects were excluded based on >5% missing genotypes and gender errors. We used linkage disequilibrium (LD) based SNP pruning to select the most informative SNPs (R\textsuperscript{2}<0.2), only for the subsequent quality control step. This resulted in ~67k SNPs for the sets to assess heterozygosity (F<3SD), homozygosity (F>3SD) and relatedness by pairwise IBD values (pihat>0.15). Datasets were merged with Hapmap Phase 3 individuals to check ethnicity. After these QC procedures on subjects (excluding in total 101 individuals), quality control on SNPs was performed as follows. All SNPs were filtered on missingness (>2%) and Hardy Weinberg (P>1e-6) before merging the three datasets. Four duplicates and three related sample-pairs were detected in the merged datasets (according to criteria described above) and one outlier after clustering the merged dataset. From these data, the \textit{MTHFR} genotype (rs1801133) was extracted.
In the replication sample, cannabis use was defined as current use more than an equivalent of £3 euro per week (roughly equivalent to weekly cannabis use) during the last month or longer. The monetary amount spent on cannabis has been reported as a valid proxy of exposure to Δ9-tetrahydrocannabinol (THC) 54.

Statistical analysis

Genotypes

Although significantly different enzymatic activity and thermolability were reported, overlapping profiles for the TT and CT genotypes have been described, with CC remaining as a distinct genotype 55. Therefore, and for power reasons, the MTHFR C677T polymorphism variable was dichotomized into T-allele carriers (TT and CT combined) and non-T carriers (the wild-type genotype CC) groups in the discovery sample. In the replication sample, genotypes were coded 0, 1, or 2 and modeled as a linear effect (additive genetic model) to account for different genotype distributions because it avoids small subgroup stratification 57. Deviation from Hardy-Weinberg equilibrium was tested by allele counting and χ² analysis.

Discovery sample

The interaction between the MTHFR genotype and TCEs on MDD recurrence was investigated using Cox regression, which takes into account differences in time at risk and censoring (no recurrence during the study period of 5.5 years). Half the study sample was randomly allocated to preventive cognitive therapy (CT: 8 sessions during the first 3 months after inclusion in the DELTA study). Preventive CT has a protective effect on recurrence that increases with the number of previous depressive episodes 40. To test whether this intervention modified the relation between MTHFR genotype, TCEs and recurrence, we assessed the significance of the four-way interaction of treatment condition by MTHFR genotype by TCEs by the number of previous episodes. Because neither the four-way MTHFR genotype by TCEs by treatment by previous episodes nor the three-way MTHFR genotype by TCEs by treatment interaction terms were significant, patients who were or were not randomized to receive CT were pooled for the Cox regression analyses. MTHFR, TCEs and MTHFR × TCEs were included as predictors in the model; the interaction term tests our hypothesis whether the effect of the MTHFR C677T variant (T-allele carrier, non-T carrier) is modified by TCEs (present, absent). To ensure that our results were not influenced by initial differences in group characteristics, we also reanalyzed the data adjusted for age, gender and antidepressant use by incorporating these variables as covariates in the Cox regression model. We used PASW statistics 18.0 (IBM SPSS, 2010, Chicago, IL, USA). We considered P<0.05 statistically significant.
Replication sample

To examine the interaction between the MTHFR genotype and TCEs on depressive symptoms, the total BDI score was regressed on TCEs, MTHFR genotype, their interaction and covariates using the following model: \( \text{BDI} = \beta_0 + (\beta_1 \times \text{covariate}) + (\beta_2 \times \text{rs1801133}) + (\beta_3 \times \text{TCEs}) + (\beta_4 \times \text{rs1801133} \times \text{TCEs}) \). As covariates we included cannabis use (modeled as a dichotomous indicator), age and gender. Analyses were performed in R (www.r-project.org) \(^{56}\). Continuous sum scores of the BDI and the CTQ were used.

Results

Sample characteristics

Descriptives of both samples are depicted in table 1.

Table 1: Characteristics discovery and-replication sample

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Discovery sample (n=124)</th>
<th>Replication sample (n=665)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female %</td>
<td>74</td>
<td>56</td>
</tr>
<tr>
<td>Age (range)</td>
<td>44.5 (21-63)</td>
<td>20.5 (18-40)</td>
</tr>
<tr>
<td>Depression score (^a)</td>
<td>3.6 (0-9)</td>
<td>5.4 (0-34)</td>
</tr>
<tr>
<td>Caucasian %</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>MTHFR C677T genotype (rs1801133)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T %</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>T/C %</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>C/C %</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>HWE(^b) (p value)</td>
<td>.32</td>
<td>.94</td>
</tr>
<tr>
<td>Childhood trauma (^c)</td>
<td>44</td>
<td>1.4 (1.0-4.3)</td>
</tr>
</tbody>
</table>

\(^a\) Discovery sample HDRS total score; replication sample BDI total score.

\(^b\) Hardy-Weinberg equilibrium.

\(^c\) Discovery sample % experienced traumatic childhood event(s) (TCE); replication sample, total score childhood trauma questionnaire (CTQ).

Discovery Sample

Of the 172 patients of the original trial, 137 provided DNA. Of these 35 patients, 15 (8.7%) were lost to follow-up and the remaining patients (11.6%) did not participate because of a diversity of reasons (for example, being afraid of needles, ethical issues concerning genetic study). Five patients could not be analyzed because of MTHFR C677T genotyping failure (genotyping success rate=96.3%). Of the remaining 132 patients, 1 was non-Caucasian, 2 were lost to follow-up and
we could not obtain TCEs data for 4 patients. All analyses pertain to the resulting 124 patients. These 124 patients were comparable to the other 48 patients on gender, age, educational level, number of previous episodes and age of onset of first depression, but differed on marital status and antidepressant use. Compared with the 48 excluded patients, the 124 remaining patients comprised fewer singles (37% vs. 54%; $\chi^2=4.14$ df=1, $P=.042$), and more users of antidepressants (57% vs. 35%; $\chi^2=6.61$ df=1, $P=.010$).

The *MTHFR* C677T genotype counts and frequencies in the patients were 60 (48.4%) for the CC variant, 49 (39.5%) for the CT variant and 15 (12.1%) for the TT variant. No deviation from Hardy-Weinberg Equilibrium was observed ($\chi^2=1.00$ df=1, $P=0.317$). T-allele carriers and non-T-allele carriers were largely comparable on demographic and psychopathological characteristics (Table 2), with the exception of educational level, and body mass index. T-allele carriers comprised more persons with medium-level education and had a higher mean body mass index, whereas non-T-allele carriers comprised more individuals with higher-level education.

Table 2: Demographic and clinical characteristics of the discovery sample (Delta) $^a$

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>T-carriers (n=64)</th>
<th>Non-T-carriers (n=60)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>Mean 16/48</td>
<td>Mean 16/44</td>
<td>.832</td>
</tr>
<tr>
<td>Age, year</td>
<td>Mean 44.0</td>
<td>Mean 45.1</td>
<td>.534</td>
</tr>
<tr>
<td>Educational level$^b$</td>
<td>Mean 34</td>
<td>Mean 32</td>
<td>.020</td>
</tr>
<tr>
<td>Low, %</td>
<td>Mean 39</td>
<td>Mean 20</td>
<td></td>
</tr>
<tr>
<td>Middle, %</td>
<td>Mean 27</td>
<td>Mean 48</td>
<td></td>
</tr>
<tr>
<td>High, %</td>
<td>Mean 42</td>
<td>Mean 32</td>
<td>.226</td>
</tr>
<tr>
<td>Marital status (single) %</td>
<td>Mean 48</td>
<td>Mean 58</td>
<td>.270</td>
</tr>
<tr>
<td>Received cognitive therapy, %</td>
<td>Mean 56</td>
<td>Mean 58</td>
<td>.815</td>
</tr>
<tr>
<td>AD use, yes %</td>
<td>Mean 28.0</td>
<td>Mean 26.0</td>
<td>.045</td>
</tr>
<tr>
<td>BMI</td>
<td>Mean 3.8</td>
<td>Mean 3.5</td>
<td>.638</td>
</tr>
<tr>
<td>HDRS17 score</td>
<td>Mean 4.5</td>
<td>Mean 7.0</td>
<td>.138</td>
</tr>
<tr>
<td>Number of previous episodes</td>
<td>Mean 29.4</td>
<td>Mean 12.7</td>
<td>.510</td>
</tr>
<tr>
<td>Age of first onset, years</td>
<td>Mean 63</td>
<td>Mean 75</td>
<td>.150</td>
</tr>
<tr>
<td>Psychiatric diseases first relatives (%)</td>
<td>Mean 40.6</td>
<td>Mean 46.7</td>
<td>.498</td>
</tr>
</tbody>
</table>

Table legend can be found on the next page >
Table 1 reports the distribution of demographic characteristics, childhood maltreatment, BDI and the MTHFR C677T genotype from individuals in the replication sample. Characteristics in the replication sample generalized to the full sample that includes all nongenotyped individuals. Genotyping was successful in all subjects and no departure from Hardy-Weinberg Equilibrium was detected.

Recurrence in the discovery sample
Overall, 98 patients (79.0%) experienced relapse/recurrence at least once over the 5.5 years. Mean time to recurrence was 750 days (SE=61.7 with a median of 493 days (range 20-2056 days)). The MTHFR C677T by TCE interaction predicted time to recurrence (P=0.017). This indicates that the predictive effect of TCEs on MDD recurrence was modified by MTHFR genotype (T-allele carriers; Table 3). This result did not change after adjusting for age, gender and antidepressant use (P=0.016).

Table 3: Effect of MTHFR modified by TCE in the discovery sample

<table>
<thead>
<tr>
<th></th>
<th>b</th>
<th>se_b</th>
<th>Wald</th>
<th>df</th>
<th>p</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR</td>
<td>-.081</td>
<td>.284</td>
<td>.080</td>
<td>1</td>
<td>.777</td>
<td>.923</td>
</tr>
<tr>
<td>TCE</td>
<td>.368</td>
<td>.296</td>
<td>1.544</td>
<td>1</td>
<td>.214</td>
<td>1.445</td>
</tr>
<tr>
<td>MTHFR * TCE a</td>
<td>.979</td>
<td>.410</td>
<td>5.713</td>
<td>1</td>
<td>.017</td>
<td>2.663</td>
</tr>
</tbody>
</table>

a P=.016 after adjustment for age, gender and AD use Cox-regression analysis.

Abbreviations - MTHFR = methyltetrahydrofolate polymorphism; T-allele carrying patients versus non-T-allele carrying patients, with non-T-allele carriers as the reference category; TCE = experienced TCE yes/no, with no TCE as the reference category;

The extent of the effect modification can be seen by comparing the risk for recurrence between the four MTHFR C677T by TCE combination groups (T+ and TCE+ N=26; T+ and TCE− N=38; T− and TCE+ N=28; T− and TCE− N=32). We found a significantly higher hazard for the T+ and TCE+ groups as compared with the T− and TCE− groups (hazard ratio 3.55; Wald 17.7, df=1, P<0.001). For patients who experienced TCEs, the hazard for recurrence in T-allele carriers...
was 2.4 times higher than in non-T-allele carriers ($P=0.002$; Figure 1). This corresponds to the observed differences in median time till recurrence that were respectively 191 days for T+ and TCE+ patients; 461 days for T− and TCE+ patients; 773 days for T+ and TCE− patients and 866 days for T− and TCE− patients.

**Figure 1**

The effect of the gene-environment interaction between *MTHFR* and TCE on time to recurrence in 124 euthymic patients with recurrent MDD over 5.5 years.

T+TC+ vs. T-TCE− = 3.55 ($P<.001$)
T+TCE− vs. T-TCE− = 0.92 ($P=.78$)
T-TCE− vs. T-TCE− = 1.45 ($P=.21$)

Relative risk for recurrence of MDD calculated with Cox-regression analysis.

**Abbreviations** - T+ = T-allele carriers; T− = non-T-allele carriers; TCE+ = experienced Traumatic Childhood Events, TCE− = no experience of Traumatic Childhood Events.
Depressive symptoms in the replication sample
Table 4 shows the results of the linear regression model in the replication sample. Significant effects on depressive symptoms were present for childhood maltreatment ($\beta = 11.08, P=5.7 \times 10^{-11}$), gender ($\beta =-1.06, P=0.036$), cannabis use ($\beta = 1.25, P=0.027$), and the $MTHFR$ genotype ($\beta = 4.13, P=0.0054$). Moreover, there was a significant interaction between childhood maltreatment and the $MTHFR$ genotype ($\beta =-3.19, P=0.0027$). For individuals carrying the TT genotype, childhood maltreatment resulted in increased levels of depressive symptoms (Figure 2).

Table 4: The effects of childhood maltreatment, and the $MTHFR$ genotype and their interaction on depressive symptoms in the replication sample

<table>
<thead>
<tr>
<th></th>
<th>b</th>
<th>se_b</th>
<th>Wald</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-9.00</td>
<td>2.98</td>
<td>-3.02</td>
<td>0.0026**</td>
</tr>
<tr>
<td>Gender</td>
<td>-1.06</td>
<td>0.50</td>
<td>-2.10</td>
<td>0.036*</td>
</tr>
<tr>
<td>Age</td>
<td>-0.023</td>
<td>0.091</td>
<td>-0.26</td>
<td>0.80</td>
</tr>
<tr>
<td>Cannabis</td>
<td>1.25</td>
<td>0.56</td>
<td>2.22</td>
<td>0.027*</td>
</tr>
<tr>
<td>Childhood maltreatment</td>
<td>11.08</td>
<td>1.66</td>
<td>6.66</td>
<td>5.7 \times 10^{-11}***</td>
</tr>
<tr>
<td>$MTHFR$ genotype</td>
<td>4.13</td>
<td>1.48</td>
<td>2.79</td>
<td>0.0054**</td>
</tr>
<tr>
<td>Maltreatment $\times$ $MTHFR$ genotype</td>
<td>-3.19</td>
<td>1.06</td>
<td>-3.03</td>
<td>0.0026**</td>
</tr>
</tbody>
</table>

* P<0.05.
** P< 0.01.
*** P<0.001.

To ensure that cannabis use in the replication sample did not influence the interaction between the $MTHFR$ genotype and childhood maltreatment, additional stratified analyses were carried out in noncannabis users ($N=478$) and cannabis users ($N=187$).

In both subsamples of the replication sample, the $MTHFR \times$ maltreatment interaction was present with a similar directionality ($P=0.050$ and $P=0.056$). Moreover, cannabis use did not interact with the $MTHFR$ genotype in the replication sample ($P=0.85$). Therefore, it is unlikely that cannabis use affected the interaction between childhood maltreatment and the $MTHFR$ genotype.

In both the discovery and replication samples, no significant association was found between the genotype and childhood maltreatment, making the presence of gene-environment correlations unlikely.
Figure 2
The gene-environment interaction between MTHFR genotype and TCEs on depressive symptoms in 665 individuals from the general population (P=0.0027). 0=T/T genotype, 1=C/T genotype, 2=C/C genotype.

Discussion
This study shows that remitted recurrently depressed patients with the thermolabile variant of the MTHFR genotype who have experienced TCEs have a poor prognosis over a follow-up period of 5.5 years, in terms of recurrence of depression. This finding supports existing evidence on the specific role of gene-environment interactions in recurrent depression, especially when TCEs are examined and the overall reported unfavorable course in patients with childhood trauma. It may explain part of the vulnerability for recurrences in MDD. In support, we also found a significant interaction between childhood trauma and the MTHFR genotype on depressive symptoms in an independent sample from the general population, underscoring the overall importance of MTHFR as a genetic risk factor for depression in the context of early-life stress.

This impact of the combination of early childhood trauma and C677T MTHFR polymorphism on onset of depressive symptomatology and recurrences in MDD gives rise to hypotheses about the underlying pathophysiological pathways. The thermolabile variant of the MTHFR gene may represent a genetic vulnerability factor for limited defense against (oxidative) stress, because
it results in a reduction of methyl donors for essential methylation processes, for example, glutathione production and synthesis of neurotransmitters. This vulnerability becomes exposed when triggered by enhanced environmental stress such as childhood trauma. This could be the result of long-lasting trauma-induced epigenetic changes. These changes include DNA methylation and chromatin modifications, patterns that are inherited but responsive to environmental shifts such as stress, and especially vulnerable during development. McGowan et al. showed altered methylation of the promoter region of the glucocorticoid receptor gene in hippocampus tissue from suicide victims with a history of childhood abuse. Interestingly, Shalev et al. recently reported stress-related accelerated telomere erosion already in childhood; compared with their counterparts, children who experienced two or more kinds of violence exposure showed significantly more telomere erosion. The authors suggest that these effects are mediated by oxidative stress. Heim et al. proposed that many of the biological changes thought to be characteristic of MDD might, in fact, be secondary to early-life trauma and represent the risk of developing MDD. Moreover, Nanni et al. revealed that childhood maltreatment was associated with lack of response (or remission) during treatment for MDD. Hypothetically, TCEs disrupt the physiological response to stress, the overactivation of which may lead to detrimental consequences in stress-sensitive systems, namely the nervous, immune, metabolic and endocrine systems. The resulting cumulative biological ‘weight’ might determine poor prognosis in terms of recurrences in MDD. However, thus far, prospective studies in recurrent MDD were lacking.

The observed gene-environment interaction is of clinical importance, as the burden of MDD is mainly because of its lifelong recurrent nature. The T− and TCE− patient groups remained on average recurrence free for 1.85 years longer than the T+ and TCE+ patient groups. This suggests that MDD patients with a childhood trauma history and carriers of the thermolabile variant of the MTHFR gene constitute a subgroup of patients who may particularly require tailored interventions. Those interventions have to combat both MDD recurrence and the consequences of childhood trauma. The gene-environment interaction in two independent samples suggests benefit from the integration of two types of therapeutic approaches: on one hand, psychotherapeutic interventions specifically aimed at the consequences of TCEs (including psychotherapeutic treatment of trauma-related problems) that, on the other hand, could be combined with interventions aimed at the 1-C cycle; (oxidative) stress may be corrected by improving antioxidant defenses through dietary modification and (add-on) exercise. These interventions should be investigated further with randomized controlled trials in specific high-risk groups.
The limitations of our study include the assessment of TCEs with a self-report questionnaire rather than an interview. Those questionnaires may be subject to recall bias through the effects of depressive symptomatology, and therefore the validity of such an approach may be reduced. However, this effect may be limited in the discovery sample because at baseline all participants were euthymic. Moreover, in the replication sample, all individuals were healthy. Furthermore, other constructs of TCEs (such as parental neglect, bullying) than those represented in the used questionnaire could play a role. In addition, the questionnaire provides no information regarding the specific timeframe in which the TCEs took place and how prolonged they were. It could be that the effect of TCEs is modified by these time-dependent factors. Another limitation in the discovery sample is the possibility of selection bias because the final sample contained 124 patients of the 172 patients from the original trial. We lost 8.7% to follow-up and 19.2% to a diversity of reasons (for example, genotyping failure, being afraid of needles, ethical issues concerning genetic study). However, the patients of the final sample did not differ on the main clinical variables from those excluded from the analyses. This makes it unlikely that this selection of 124 patients out of the original sample induced any additional selection bias. Similarly, in the replication sample, we cannot exclude that recruitment strategies have resulted in a sample that is not completely representative of the general population with regard to age and educational level, and therefore we cannot be sure that the findings in the replication sample can be generalized to the general population.

In spite of these limitations, our study is unique in providing the opportunity to investigate the role of the interaction between genes and environment on prospectively assessed recurrence over 5.5 years. In addition, this was investigated in a specific sample of highly recurrent depressed patients, which can be considered characteristic for those patients particularly causing the large MDD-associated burden of disease. By including specific recurrently depressed patients, we were able to investigate a clinically highly relevant sample. Our replication of the interaction in a population sample supports the robustness of our findings and suggests that this genetic vulnerability is relevant in the broader context of depression.

In summary, the results of the present study indicate that an interaction between \textit{MTHFR C677T} and TCEs increased risk of recurrence in recurrent MDD patients over 5.5 years of follow-up and is associated with depressive symptoms in the general population. More attention to specific at-risk individuals, that is, patients who experienced TCEs and genetic alterations, including \textit{MTHFR C677T}, could help to improve treatment strategies to prevent depression and recurrences. However, the exact nature of the connection between \textit{MTHFR C677T}, TCEs and the
course of recurrence in MDD remains to be clarified. Future, preferably prospective, studies are warranted to replicate these findings.

Acknowledgments
This study has been made possible due to financial aid of the Netherlands Foundation for Mental Health, Utrecht and the Health Research Development Council, Department Prevention Program (ZONMw). The replication study was supported by a grant of NWO, the Dutch council for scientific research (ZonMW TOP grant no. 91207039). Funders had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

We are most grateful to the participants of our study. In addition, we express our appreciation to the participating psychiatric sites for their recruitment efforts. We also thank our interviewers and independent raters and specifically Irene Visch for assistance with data management and support. The following colleagues contributed to the DELTA (Depression Evaluation Longitudinal Therapy Assessment) Study: Mascha ten Doesschate, Jochanan Huyser, Guido Nabarro, Philip Spinhoven, Ellie Wekking and Luuk Wouters. We also like to thank Chris Schubart and Willemijn van Gastel for their input in the replication sample.

Declaration of Interest
The authors declare no conflicts of interest. AL had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Time after Time: biological factors in the course of recurrent depression

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Time after Time; biological factors in the course of recurrent depression


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Chapter 6
Medically unexplained physical symptoms
Medically unexplained physical symptoms

6.1 Sustained medically unexplained physical symptoms in euthymic patients with recurrent depression: predictive value for recurrence and associations with omega 3- and 6 fatty acids and 5-HTTLPR?

Lok A, Assies J, Koeter MWJ, Bockting CLH, Wouters LF, Mocking RJT, Schene AH

Abstract

Background
Identification of potentially modifiable risk factors for recurrence in recurrent depression could provide opportunities to improve preventive interventions. In this study we aimed to examine the predictive value of medically unexplained physical symptoms (MUPS) on time to recurrence in recurrent depression. Additionally, to elucidate pathophysiological mechanisms that could explain the relations between MUPS and depression, we investigate the association between a sustained high level of MUPS, and (I) omega (ω)-3 and − 6 fatty acid (FA)-status and (II) functional polymorphisms in the promoter region of the serotonin transporter gene (5-HTTLPR).

Methods
Based on three Physical Symptom Checklist (PCS) scores over 12 months, we defined two groups of remitted recurrently depressed patients: 41 patients with a sustained high number of MUPS and 34 patients with a sustained low number or no MUPS. Patients were followed-up for 3.5 years while recurrence of their depression was monitored. In addition, we analyzed patients’ erythrocyte’s FA-profiles and triallelically genotyped their 5-HTTLPR.

Results
A sustained high level of MUPS predicted consecutive depression recurrence over 3.5 years (adjusted relative risk 2.8). FA-status and distribution of 5-HTTLPR variant frequencies did not differ between patients with sustained high compared to low/absent MUPS-levels.

Limitations
Our sample was relatively small.

Conclusion
Remitted recurrently depressed patients with sustained MUPS have a considerably increased risk of recurrence. Having sustained MUPS is not associated with either erythrocyte ω – 3 or − 6 FA-levels or 5-HTTLPR polymorphism. Recognition and reducing MUPS in an early state could prevent a (depressive) relapse.
1. Introduction
Many patients (40-50%) with depression will have more than one episode and therefore suffer from the recurrent type of this disorder. Identifying predictors for recurrence in these patients is important for a better understanding of the course of this disease. Well known risk factors for recurrence are: clinical variables (age at onset of first episode, severity of first episode, number of previous episodes, residual symptoms), family history, negative/extreme cognitions, personality (neuroticism), daily hassles, poor social support and maladaptive coping styles. These predictors explain only part of the variation in recurrence. Identification of dynamic, potentially modifiable, risk factors for recurrence in particular could provide an opportunity for developing targeted preventive intervention.

Somatic symptoms, not attributable to a diagnosable medical condition, are common in patients with depression. These so-called medically unexplained physical symptoms (MUPS) show a wide variety of severity, ranging from a single, mild and transient symptom to a larger number of more chronic and extremely debilitating ones. Insight in the relation between MUPS and depression is interesting because high levels of MUPS in depressed patients may (I) greatly harm their quality of life and increase the burden of depression, (II) hinder full remission, and (III) impede treatment response. Hence, the need for proper recognition of MUPS and appreciation of their clinical value is unquestionable.

Cross-sectionally, the correlation between depression and MUPS has been well established. However, longitudinal studies examining the temporal relationship between depression and MUPS are scarce. It has been postulated that MUPS, instead of fluctuating in severity parallel to the depressive symptoms, remain present in between depressive episodes. Therefore, MUPS could be regarded as (I) residual symptoms of a depressive episode, and/or (II) an early indicator of, or a risk factor for a new depressive episode. Identifying MUPS as a predictor of recurrence in patients with recurrent depression could be clinically relevant, also because MUPS may represent a dynamic modifiable factor involved in recurrence.

The relation between depression and MUPS may be due to shared underlying biological pathways. In this paper we focus on two of these related pathways. First, the polyunsaturated fatty acid (PUFA)-metabolism, because PUFAs (I) participate in immune regulation (II) determine neuronal membrane stability, and (III) are involved in neurotransmission and signal transduction. Depression is associated with lowered omega-3 (\(\omega-3\)) fatty acid levels and an imbalance between \(\omega-3\) and \(\omega-6\) PUFAs, which is generally being hypothesized as
harmful\textsuperscript{24,22} and might be linked to somatic manifestations\textsuperscript{23}. Second, another possible underlying mechanism may be serotonergic pathways, because they are considered to play a role in both depression and the development of physical (pain) symptoms\textsuperscript{19}. There is evidence for an association of longer \textit{5-HTTLPR} allele mutations with MUPS, while depression itself is associated either with a longer \textit{5-HTTLPR} allele mutation or other \textit{5-HTTLPR} mutational variants\textsuperscript{25,26,27}. Lowered omega-3 FA status is related to serotonergic disturbances which offer an etiological pathway for mood and cognitive dysfunction in depression\textsuperscript{23}.

No study thus far has examined the impact of MUPS on the prognosis of recurrence in the recurrent type of depression. Therefore, the first aim of our study was to determine the predictive value of a sustained high level of MUPS for recurrence in euthymic patients with recurrent depression. Second, we aimed to determine whether the biological profiles of the patients with and without a sustained high level of MUPS differ. We hypothesized that a high level of MUPS would be accompanied by an increased $\omega - 6/\omega - 3$ FA ratio and a higher expressing variant of the \textit{5-HTTLPR} polymorphism.

2. Methods
2.1. Study population
The current study was part of the DELTA-study, a randomized clinical trial, investigating the effect of cognitive therapy on recurrence in euthymic patients with $\geq 2$ previous major depressive episodes (MDEs) in the last 5 years. Among the exclusion criteria were current or previous mania or hypomania (bipolar disorder), any psychotic disorder (current or previous), alcohol or drug abuse and predominant anxiety disorder. Participants were recruited from psychiatric centers and through media announcement. Recurrence of depression was the main outcome parameter. Since neither type of aftercare, nor AD use, was an inclusion or exclusion criterion for the study, with respect to these characteristics, the DELTA sample can be considered representative for patients suffering from recurrent depression. All patients provided informed consent to enter the protocol which was approved by the institutional ethics review committees. The background and methodology of the DELTA-study is described in more detail previously\textsuperscript{28}.

For our MUPS study two periods of the DELTA-study are of importance (see Fig. 1). The first one, month 12 to month 24 of DELTA, was used to define the MUPS group and includes three moments of assessment: 12 (T$-12$), 18 (T$-6$) and 24 (T$0$) months after inclusion. The second is the period in which we assessed recurrence. This follow up starts at month 24, defined as T$0$, and runs for 3.5 years.
The DELTA-baseline sample comprised 187 participants of which 15 were excluded because they dropped out of the study immediately after randomization. From the remaining 172 patients we included those patients (a) who attended the study-protocol at T− 12, T− 6 and T0, (b) had a valid Physical Symptom Checklist (see further) at T− 12, T− 6 and T0, (c) who were euthymic (had no DSM IV-R diagnosis of major depressive episode) in the period between T− 12 and T0, and (d) had at least one follow up measurement after T0 (time at risk > 0). The latter two criteria were necessary requirements for standard Cox regression analysis. Of the 187 patients, 78 patients did not fulfill one or more of the above criteria (24 criterion b, 47 criterion c, and 7 criterion d), resulting in 109 eligible patients.

2.2. Measures

2.2.1. Medically unexplained symptoms

Physical symptoms were assessed three times (T− 12, T− 6, T0) using the Physical Symptom Checklist (PSC). The PSC is a self-report checklist comprising 55 physical symptoms covering most organ systems. Four of these 55 are gender specific (one for men and three for women). These items were excluded to rule out bias by gender. From the 51 remaining items, 10 items measure autonomous symptoms, 11 are general/neurological, 8 musculoskeletal/pain, 13 gastrointestinal, 5 urological/genital items and 4 items are about feeling hot/cold. Each symptom is rated by the patient on a 4-point Likert scale (0-3) with the preceding week as the time frame. For the analyses, each symptom score is dichotomized into ‘not present’ (scores 0 and 1) or ‘present’ (i.e. ‘bothersome often or most of the time during the previous week’, scores 2 and 3). The resulting total symptom score ranges from 0 to 51 and can be considered an indicator of the severity of somatoform disorders and a predictor of health care utilization.

At the same time as the PCS assessments, patients were asked in an open interview about their concurrent physical illnesses. Two clinicians (AS and JA), blind to treatment and relapse/recurrence, compared the results from the PSC and the interviews. When a physical illness
or complaint mentioned by the patient could explain individual items of the PSC, these items were recoded as 'not present'. The remaining items of the PSC which could not be explained by a physical illness were considered 'medically unexplained physical symptoms’. When doubt remained (i.e. 21 physical complaints), the symptom was regarded as 'explained'.

2.2.2. Definition of MUPS groups

Based on the PSC scores at T− 12, T− 6 and T0 we defined two patient groups to maximize the contrast: (1) a MUPS + group comprising 41 patients with a sustained high number of MUPS (i.e. a PSC score ≥ 5 at each of the three assessments), and (2) a MUPS − group, comprising 34 patients with a sustained low number or no MUPS (i.e. a PSC score < 5 at each of the three assessments). This threshold of 5 was based on Escobar’s abridged somatization construct SSI 4/6 30, 33. The presence of high levels of symptoms (e.g. ≥ 5) was suggested as a reasonable threshold to designate “cases” in clinical and epidemiological studies. The remaining intermittent group of 34 patients had 5 or more MUPS at some of the 3 assessments and less than 5 MUPS at the other assessments and was excluded from the analyses. In summary, both the MUPS + and MUPS − groups did not suffer from a depressive episode during the assessment of the MUPS, and the MUPS that defined that those groups could not be explained by any known physical condition.

2.2.3. Relapse/recurrence

To assess relapse/recurrence, we used the Structured Clinical Interview for DSM-IV (SCID-I) 34 at 3.5 years follow-up (T42: the end of the study). At assessment, current and past depressive episodes were checked by trained SCID evaluators who were blind to treatment condition. Subjects were instructed not to reveal treatment condition to the interviewers (psychologist/research assistants). All interviews were audio taped. Two independent experienced psychiatrists, blind to treatment condition, evaluated all occasions of participants meeting the DSM-IV criteria for MDD. In cases of disagreement, the ratings of the psychiatrists were used. Kappa for inter-rater agreement between the interviewers and psychiatrist on categorization of a relapse/recurrence or no relapse/recurrence was .96, indicating high agreement.

2.2.4. Sample collection of fatty acids

Fatty acids in washed erythrocytes were analyzed at T0 using capillary gas chromatography, described previously 2. Samples were stored at – 80 °C until analysis.
2.2.5. Genotyping procedures and analysis

For genotyping, two venous blood samples of 10 ml were taken at T0, mixed with EDTA to prevent coagulation, and stored at room temperature within 12 h after sampling, until analysis.

Genomic deoxyribonucleic acid (DNA) was isolated using a filter-based method (QIAamp DNA Mini Kit, Qiagen Ltd, United Kingdom). The length of the 5-HTTLPR polymorphism was determined by gel electrophoresis. The region around the polymorphism was amplified by PCR using forward primer \texttt{tgtaaacgacggcagtgccagcaccctaat} and reverse primer \texttt{caggaacacgtatgccagggagatctgggaga} (M13 primer sequence in italic). The PCR reaction was performed in 10 μl containing 1.5 mM MgCl2, 0.2 μM forward and reverse primer, 0.1 mM dNTPs, 0.5 Units Hotfire Polymerase (Solis Biodyne, Estonia), Buffer B (Solis Biodyne, Estonia) and 20 ng genomic DNA. The lengths of the four different alleles were: short = 250 bp, long = 298 bp, long + = 320, long ++ = 380 bp. Genotyping of the rs25531 SNP was done by sequencing (Sanger) using Big Dye Terminators (Applied Biosystems). The M13 forward primer \texttt{tgtaaacgacggcagtgccagcaccctaat} was used for sequencing. 10 μl reactions were performed containing 5 ng of a forward primer, 5 μl PCR product, BDT mix (Applied Biosystems) and 2.5 × BDT buffer (Applied Biosystems). The length of the 5-HTTLPR polymorphism was confirmed by looking at the length of the sequenced PCR product.

2.3. Statistical analysis

Analyses were performed in SPSS statistics 18.0 (SPSS, Inc., 2009, Chicago, IL). The effect of MUPS on recurrence was assessed with Cox regression, which takes into account differences in time at risk and censoring (no recurrence during the study period). To maximize the contrast we restricted the analyses to the MUPS + and MUPS − patient groups.

Half of the study sample randomly received 8 sessions of cognitive therapy during the first three months after inclusion in the DELTA study. This therapy prevented recurrence and its preventive effect increased with the number of previous depressive episodes. To test whether this intervention modified the relation between MUPS and recurrence we assessed the significance of the 3-way interaction of treatment condition by high MUPS by number of previous episodes’ interaction. Because neither the 3-way MUPS by treatment by previous episodes nor the 2-way MUPS by treatment interaction terms were significant, both experimental and control groups were pooled for the Cox-regression analyses. Log-log survival plots showed that the proportional hazard assumption was met.
To examine whether initial differences in relevant variables confounded the effect of sustained MUPS, estimates were adjusted for the potential confounding effects of the following variables by incorporating them as covariates in the Cox regression analyses: gender, marital status, previous depressive episodes, early onset of depression (in years), antidepressant use (yes/no, continuous yes/no); monitored with the Trimbos/IMTA Self Report Questionnaire for Costs Associated With Psychiatric Illness [TIC-P] and assessed information on AD (type and dosage) every 3 months during follow-up, life events (Negative Life Events Questionnaire), Personality Disorder Questionnaire total score (PDQ-4+) and the 90 item Symptom Check List total score (SCL-90)\textsuperscript{35, 36, 37, 38}.

The triallelic genotypes were reclassified into a biallelic model by their levels of expression. We grouped the genotypes as S'/S' (S/S, L_d/S, L_d/L_d), S'/L_A (S/L_A, L_d/L_A) and L_A/L_A. In the analyses we combined S'/S' and S'/L_A genotypes to compare them with the high-expressing L_A/L_A genotype\textsuperscript{39}.

Fisher’s exact test was used to examine the significance of the association (contingency) between the two kinds of MUPS classification regarding the biallelic model. Independent means t-tests were performed to detect differences in FA concentrations between patients with and without sustained high levels of MUPS.

3. Results
The 75 patients (of the 172) comprising the MUPS groups were comparable to the other 97 patients on gender, educational level, marital status, % Caucasian, AD use, number of previous episodes, and age at onset of first depression, but differed on current age. The MUPS group was on average 3 years older (MUPS groups patients mean age 46.4, sd 8.2; other patients’ mean age 43.4, sd 10.2; \( t = 2.14 \text{ df} = 170, p = .033 \)).

Characteristics of both MUPS groups are summarized in Table 1, which shows that both groups were comparable on the variables tested (all \( p \)'s > .10), except there were more women in the MUPS + group.
Table 1: Characteristics of recurrently depressed patients with high and low levels of medically unexplained physical symptoms (MUPS) at baseline

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n = 75)</th>
<th>MUPS + group (n = 41)</th>
<th>MUPS − group (n = 34)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (%)</td>
<td>73</td>
<td>85</td>
<td>59</td>
<td>.009</td>
</tr>
<tr>
<td>Age (yr, mean, (SD))</td>
<td>46.4 (8.2)</td>
<td>46.8 (9.0)</td>
<td>45.9 (7.3)</td>
<td>.638</td>
</tr>
<tr>
<td>White (%)</td>
<td>98.7</td>
<td>100</td>
<td>97</td>
<td>.325</td>
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<tr>
<td>Single (%)</td>
<td>40</td>
<td>44</td>
<td>35</td>
<td>.456</td>
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<tr>
<td>BMI (kg/m2) (mean (SD))</td>
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<td>26.4 (4.5)</td>
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<tr>
<td>Antidepressant medication (%)</td>
<td>59</td>
<td>63</td>
<td>45</td>
<td>.127</td>
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<tr>
<td>Age of first onset (yr, mean (SD))</td>
<td>30.3 (12.7)</td>
<td>29.7 (14.3)</td>
<td>31.0 (10.6)</td>
<td>.643</td>
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<td>Previous episodes (median (SD))</td>
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<td>7.1 (11.1)</td>
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<td>.145</td>
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All percentages χ² test and all continuous variables t tests.

Over the consecutive 3.5 year follow-up period (month 24 [T0] to month 66 [T42] of the original trial) time to recurrence was significantly longer for patients belonging to the MUPS − group (Fig. 2). In other words, euthymic patients without sustained MUPS stayed significantly longer in remission than patients who suffered from MUPS, a result which holds, both with and without correction for potential confounders. Compared to the MUPS − group, the MUPS + group had a raw relative risk of 2.6 (Wald(1) = 9.38, p = .002) and an adjusted relative risk of 2.8 to experience a recurrence.

Physical complaints most frequently mentioned by patients in the MUPS + group were: pain in the extremities (54% of patients), muscle tension (54%), back pain (51%), shortness of breath, (49%), nausea, (41%) and headaches (37%).

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Figure 2:

Recurrence of new depressive episodes in patients with low and high levels of medically unexplained physical symptoms (MUPS).

There were no significant differences in total $\omega - 3$ and $\omega - 6$ PUFAs and total fatty acids between the MUPS + and MUPS − group (Table 2). The concentrations of C20:4$\omega - 6$ (arachidonic acid; AA), C20:5$\omega - 3$ (eicosapentaenoic acid; EPA), total $\omega - 6/\omega - 3$, AA/EPA and C20:4$\omega - 6$/C22:6$\omega - 3$ (AA/DHA) ratios were comparable between both MUPS groups.

Table 2: Erythrocyte fatty acid concentrations (pmol/10e6 erythrocytes) of recurrently depressed patients with high and low levels of medically unexplained physical symptoms (MUPS)

<table>
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<tr>
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<th>MUPS + group (n = 31)</th>
<th>MUPS − group (n = 20)</th>
<th>$p$ value</th>
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<tr>
<td>Eicosapentaenoic acid (C20:5 $\omega - 3$)</td>
<td>3.3 ± 1.1</td>
<td>3.2 ± 1.2</td>
<td>.887</td>
</tr>
<tr>
<td>Docosahexaenoic acid (C22:6 $\omega - 3$)</td>
<td>14.8 ± 3.6</td>
<td>14.8 ± 3.8</td>
<td>.992</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4 $\omega - 6$)</td>
<td>70.4 ± 7.4</td>
<td>70.9 ± 8.4</td>
<td>.841</td>
</tr>
<tr>
<td>C20:4 $\omega - 6$/C20:5 $\omega - 3$ (AA/EPA)</td>
<td>24.3 ± 8.6</td>
<td>25.6 ± 11.3</td>
<td>.659</td>
</tr>
<tr>
<td>C20:4 $\omega - 6$/C22:6 $\omega - 3$ (AA/DHA)</td>
<td>5.0 ± 1.1</td>
<td>5.0 ± 1.2</td>
<td>.984</td>
</tr>
<tr>
<td>Total $\omega - 6/\omega - 3$</td>
<td>6.1 ± 1.2</td>
<td>6.1 ± 1.2</td>
<td>.940</td>
</tr>
<tr>
<td>Total fatty acids</td>
<td>588 ± 51</td>
<td>579 ± 59</td>
<td>.619</td>
</tr>
</tbody>
</table>

Independent means t tests.
Genotype distribution was in Hardy-Weinberg equilibrium in the triallelic ($\chi^2 (3) = 4.33, p = .44$) but not in the biallelic ($\chi^2 (1) = 4.20, p = .04$) model. However, when we stratified the biallelic model into MUPS $+$ [$\chi^2(1) = 3.55$, $p = .06$] and MUPS $-$ [$\chi^2 (1) = 1.02, p = .31$] groups genotype distribution was in Hardy-Weinberg equilibrium in each stratum. Analyses of serotonin transporter gene promoter polymorphisms in recurrently depressed patients with high and low levels of MUPS showed that the 20.0% of the MUPS $+$ patients and 30.8% of the MUPS $-$ patients had a $L_\alpha$ containing bi-allelic functional profile ($L'L'$ or $L'S'$). These differences did not reach statistical significance ($\chi^2 (1) = .933, p = .334$). The groups also did not differ with respect to the triallelic profile (Fisher exact test $p = .831$).

4. Discussion

The aim of this study was, first, to determine whether remitted recurrently depressed patients with medically unexplained physical symptoms (MUPS) have a poor prognosis over a follow up period of 3.5 years, in terms of recurrence of depression. During the follow up period 50% of MUPS $-$ patients and 80% of the MUPS $+$ patients experienced a new depressive episode. This suggests that a sustained high number of these symptoms over a 12 month period are predictive for subsequent recurrence. This result holds when we adjusted for confounders such as sex and the use of antidepressants, but also when we control for well known illness related predictors for recurrence, i.e. the number of previous episodes, the age of onset of depression and residual symptoms.

The way we defined and assessed MUPS was meant to ascertain as well as possible that these somatic symptoms were not explained by any physical disorder or illness known to the patient. The predictive power of MUPS could also not be explained by residual depressive symptoms as we corrected our analyses for these symptoms. MUPS were also not part of a depressive episode, because patients were free of such episodes when MUPS were assessed. Finally, it is unlikely that the predictive value of MUPS is confounded by antidepressant use, because we corrected our analysis for the use of this medication. One might argue that the reported muscular tension and shortness of breath, may in fact reflect a high level of anxiety. However, the association between MUPS and the risk of relapse could not be explained by general psychological complaints, including anxiety symptoms. Moreover patients with premorbid anxiety disorders and substance related disorders were excluded in this study. So, the reported MUPS correspond to symptoms that are possibly not related to these often co-morbid disorders.

Somatic complaints represent a risk factor for the subsequent development of depressive symptoms in nonclinical populations $^{40, 41, 42}$. In clinical populations of depressed patients,
physical symptoms, such as pain, are well documented and common \(^{43, 44}\), and may be of greater importance to patients than the mood disturbance \(^{45}\). About half of the patients in general practice with depression suffer from a somatoform disorder as well \(^{47}\). This relationship could be due to anxiety and depression causing (awareness of) physical symptoms, or physical symptoms causing anxiety and depression. Alternatively, this relationship could be explained by a more complex circular relationship \(^{29}\), and/or the existence of common (genetic) risk factors.

MUPS might theoretically develop into a new depressive episode by two collateral causal pathways. First, simply as an ongoing common pathophysiological process without any intervening components. Second, in our opinion clinically more plausible, by a set of mediating factors. Symptoms of e.g. pain, muscle tension and headache may interfere with access to positive reinforcers (i.e. enjoyable activities), lead to increased feelings of helplessness, and activate negative cognitive schemas or distortions (e.g. catastrophizing) that contribute to depression \(^{46, 20}\). Activated depressive cognitions may contribute to a further misinterpretation of physical sensations as being indicative of an underlying illness \(^{47, 48}\).

Alternatively, MUPS and depression can be considered as two distinct disorders with a common pathogenetic basis. This basis could be related to the ubiquitously held ‘monoamine theory’ of depression which holds that depression is related to alterations in key neurotransmitters, including serotonin, dopamine and noradrenalin \(^{49}\). These neurotransmitters are also involved in pain modulation, which under normal conditions functions of and to block or dampen pain signals. Consequently, any ‘alterations’ in these key neurotransmitters would be expected to affect both pain and depression, and this biological link may help explain the high rates of comorbidity between pain and depression \(^{19, 20}\).

In the present study, pain in the extremities and muscle tension were the most reported (pain related) symptoms; a finding that is consistent with previous studies frequently reporting muscle soreness in major depressive disorder \(^{46, 50, 51, 20}\). This symptom might be expected to contribute to the development of, or vulnerability to, other (pain-related) symptoms, including those measured in this study. Although these pain symptoms were accessed as separate categories, they may in fact be mediated by the same underlying factor \(^{20}\). In that case depression and MUPS share common pathways of symptom development. Interestingly, recent developments postulate that depression might be related to mitochondrial dysfunction \(^{52}\) and that decreased ATP production rates might be present in depressed patients with very high levels of somatic symptoms \(^{53}\).
Somatic symptoms are also thought to be related to higher expressing alleles (L) of 5-HTTLPR, which should facilitate higher reuptake rates of serotonin. However, also links to lower-expressing allele (S) were reported for fibromyalgia and for painful symptoms in major depressive disorder. We did not find the expected positive association between allelic variation in 5-HTTLPR and a sustained high level of MUPS in patients with recurrent depression. MUPS + patients with recurrent depression did not differ from MUPS − patients in the frequency of the functional biallelic form of 5-HTTLPR polymorphism. Unfortunately, we examined only a single candidate gene. In regard to the heterogeneity of symptoms in both MUPS and recurrent depression, interactions of multiple genes as biological bases for the psychiatric phenotypes are thought to be more likely. Other explanations for our findings could be that (I) defined our MUPS groups in terms of longitudinal, repeated symptomatology, whereas other studies used other definitions, and (II) were able to make an absolute contrast with a symptom-free MUPS group. However, the nature of the connection between 5-HTTLPR allelic variants and somatic symptoms remains to be clarified.

Depression is associated with lowered ω−3 fatty acid levels and an imbalance between ω−3 and ω−6 PUFAs. In a previous study we showed that in erythrocytes of patients with recurrent depression the concentrations of docosahexaenoic acid and arachidonic acid were lower than in healthy controls, and in addition, patients had a higher ω−3 and ω−6 ratio. In this study, MUPS + patients with recurrent depression did not differ from MUPS − patients in their ω−3 and ω−6 PUFA status and the ratios (ω−6/ω−3 AA/EPA, AA/DHA). Riemer et al. studied the fatty acid status in major depressive patients and patients with comorbid somatoform and depressive disorders. MDD patients and patients with both disorders had significant higher AA/EPA, AA/DHA and total w3/w6 in serum cholesterylesters. However, as in our study, no significant differences for the fatty acid composition of serum phospholipids were detected. We refrain from drawing firm conclusions as factors influencing fatty acid metabolism such as dietary intake of fat and fatty acids, the use of supplements, alcohol consumption, smoking habits and physical activity were not assessed systematically.

Another limitation is the difficulty to distinguish between MUPS and physical symptoms that are part of a broader psychiatric condition, such as depression. Although the selection of appropriate patients for research on MUPS is essential, this judgment may be difficult to make in clinical practice, particularly as the co-morbidity between medically unexplained symptoms, depression and anxiety is extremely high. Some researchers have tackled this by assuming that a physical symptom cannot be regarded as medically unexplained if it is one of the diag-
nostic features of major depression or panic disorder (e.g. fatigue, autonomic symptoms etc.) and full diagnostic criteria for that condition are also met. This is potentially problematic as some physical symptoms can be misdiagnosed as medically unexplained despite only being present during episodes of panic or depression that are not severe enough to meet formal criteria for these disorders. Conversely, patients often experience physical symptoms of this sort and develop a depressive disorder some time later; many of these patients are understandably reluctant to view their initial physical problems as symptoms of a ‘hidden’, ‘masked’ or ‘denied’ psychiatric illness. In a multicenter, international epidemiologic study, 69% of the patients who met the criteria for depression reported physical symptoms as their only reason for consulting a physician. However we tried to carefully define participants in terms of the presence of recurrence of depression by undertaking a detailed structured interview and to exclude those with a current relapse from our analyses. Post-hoc, we tested whether the predictive value of MUPS was caused by the fact that some MUPS can be part of depressive symptomatology, and 8 symptoms were removed from the PCS that are (part of the) criteria of depression (DSM-IV), namely; items 1 (feeling tired or having low energy), 2 (easily fatigued without exertion), 8 (sleeplessness), 9 (sleeping a lot), 10 (forgetfulness), 28 (loss of appetite), 29 (weight loss, last month) and 51 (sexual indifference). This did not affect the results.

Considering the various views on MUPS in relation to depression, there are several approaches to and opportunities of integration of different treatments. In our study, MUPS were identified as dynamic (potentially modifiable) risk factors for recurrence and could provide the opportunity to preventive intervention. It is mandatory that the somatic symptoms are recognized and reduced in an early state to prevent a (depressive) relapse. In addition, reduction of (pain) symptoms, could establish a better activity level of patients, and might thereby lower the risk of recurrence. Primary treatment of MUPS is also relevant to antidepressant treatment selection, because the mechanisms that may subserve their efficacy for treatment of MUPS are hypothesized to be related to the modulation of both serotonergic and norepinephrinergic neurotransmission. Thus far, for antidepressant treatment of MUPS, there is no clear evidence on the optimum dose, duration of treatment, or long-term outcome. In addition, there exists no firm evidence which antidepressants or other pharmaceutical agent can be regarded as the optimal approach to treat MUPS. However, studies suggest that CBT is a promising treatment for MUPS.
5. Conclusion
Sustained medically unexplained physical symptoms predict relapse and recurrence in recurrent depression. This effect seems not to be attributable to shared underlying pathological abnormalities in omega-3 or -6 FA-levels or 5-HTTLPR mutations. More attention for MUPS in patients with recurrent depression could lead to improved (preventive) treatment strategies and outcome measures.

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Conflict of interest
The authors declare that they have no (financial and non-financial) competing interests related to this work. A. Lok had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data-analysis.

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Chapter 7
General Discussion
General discussion

Summary, conclusions and general discussion

_Lok A_

_Submitted to J of Psychotraumatology to be published._
In this chapter, the conclusions of our studies will be summarized and the findings will be discussed. Possible limitations and strengths of this thesis as well as the implications for future research will be addressed.

Summary
The primary focus of this dissertation was to increase the knowledge about possible biological factors and psychopathological mechanisms (including HPA/axis functioning, one-carbon and fatty-acid metabolism), as well as some gene-environment interactions for the course of recurrent MDD (MDD-R). Studying MDDR is important because there are indications that it represents a more biological and genetic determined MDD-subtype, which may be specifically linked to recurrence and CVD-risk. For this reason we expected these patients to deviate the strongest from healthy controls in the (patho)physiological and genetic mechanisms and to have more pronounced alterations in their course of MDD.

We considered two leading hypotheses that potentially could explain the underlying mechanism for the high risk for recurrence: (I) the vulnerability-accumulation or scarring hypothesis and (II) the premorbid vulnerability hypothesis. Our aim was to investigate specific premorbid factors (e.g. genetics, childhood trauma) that are present before MDD onset as well as the specific biological factors that could play a premorbid role and/or are involved in vulnerability-accumulation (‘scarring’). These biological variables were collected in different stadia in the course of MDD-R, i.e. the remitted phase, subsyndromal phase and acute depressive state.

Studies in this thesis are mostly based on data collected from the participants of the DELTA study: a well-defined high-risk population for relapse and recurrence as it includes exclusively remitted patients with at least two previous depressive episodes.
Research questions

This thesis consists of substudies conducted to answer the following questions regarding some pathophysiological mechanisms, which possibly influence the course of MDD-R:

1. What is the relation between recurrent depression, being overweight, and obesity in patients with MDD-R and is this relation explained or modified by use of antidepressant (AD) medication?

2a. In what ways does the HPA-axis functioning differ between MDD-R patients and healthy controls?

2b. Does this reflect a persistent trait or is this influenced by depressive state, stress or previous episodes, associated with recurrence?

2c. Can this relation be modulated by cognitive therapy?

3. Do HPA-axis measures predict time to recurrence in MDD-R patients?

4a. Do polyunsaturated fatty acid (PUFA) levels and the n-6/ n-3 ratio differ between MDD-R patients compared to non-depressed controls?

4b. Are these possible alterations “state” dependent, or do they reflect a trait (i.e. are they independent of the current depressive status)?

5. What is the relationship of the Ala54Thr fatty acid-binding protein 2 (FABP2) polymorphism in MDD-R and are fatty acid concentrations associated with the CVD risk factor waist circumference?

6. What is the prevalence of the MTHFR polymorphism and its relationship with 1-C-cycle components in MDD-R patients compared to matched controls, and are the latter influenced by depressive state and/or AD use?

7. What is the relation between recurrent MDD patients carrying the T allele in the MTHFR gene with time to recurrence and is this relation associated with having been exposed to traumatic childhood events (TCE)?

8. What is the association between medically unexplained physical symptoms (MUPS) and time to recurrence in MDD-R and what is the association between MUPS and FA metabolism and MUPS and the serotonin transporter gene?

Timeline figure DELTA with assessments
Summary of the results of the studies

Obesity

In chapter 2 (research question 1) we show that overweight and obesity were more prevalent in patients with MDD-R than in the reference group, although this was only statistically significant in women (74% of the sample). MDD-R patients using ADs (mostly SSRIs) continuously showed significantly more abdominal overweight and obesity than those using ADs intermittently or not at all. Compared with SSRIs, other types of ADs used (e.g. tricyclic ADs) did not have a significant impact on the anthropometric measures. However, we did find a small positive association between AD equivalent dosage and waist circumference and waist-to-hip ratio.

This was studied in MDD-R patients (n=134) and compared with reference data of BMI, waist-to-hip ratio and waist circumference, derived from the cross-sectional monitoring project on risk factors for chronic diseases (MORGEN project), a large Dutch population-based study of time trends on obesity prevalence rates.

A better understanding of the relationship between obesity and depression, and the beneficial and adverse effects of psychotropics on appetite, eating behavior, body weight and metabolism, may improve our ability to prevent and treat both obesity and depression. This supports the development of person-tailored interventions for MDDR including effective non-pharmaceutical preventive treatment and extra physical activities which may - as added benefit - protect against AD-induced weight gain.

HPA-axis

In chapter 3 (research question 2), we describe that MDD-R patients had higher cortisol concentrations than gender and -age matched controls. This did not change during new MDD-episodes during follow-up (3 months and 2 years). HPA-axis activity was not related to daily hassles or childhood life events. Interestingly, cortisol concentrations were lower in patients with more previous episodes, but were not associated with recurrence(s) during follow-up. Finally, randomly assigned cognitive therapy at study-entry enhanced cortisol declines over the day throughout the twoyear follow-up.

These results indicate that remitted MDD-R patients have a persistent trait of increased cortisol concentrations, irrespective of stress. In combination with the finding that patients’ cortisol concentrations do not change during new MDD-episodes (and thus do not represent epiphenomenal or state-effects), our results support that hypercortisolemia fulfills the state-independence criterion for an endophenotype for MDD-R.
Moreover, in this chapter (research question 3) we addressed the observation that lower mean morning cortisol levels predicted earlier time to recurrence over 5.5 year after correction for residual symptoms. Residual symptoms and childhood trauma slightly confounded the association between cortisol and recurrence. Lower cortisol levels were associated with having experienced traumatic childhood life events (42.3% in patients with lower cortisol versus 19.2% in patients with higher cortisol).

This study provides further support for the predictive role over 5.5 year of HPA axis dysregulation (i.e. lower morning cortisol levels) for recurrence in recurrently depressed patients. Childhood trauma was associated with having lower cortisol levels. This might have long-term consequences for dealing with (psychological) stress and dealing with the accompanying HPA-axis alterations.

Fatty acid metabolism
Chapter 4.1 (research question 4 a) first describes an explorative study in which we randomly selected patients from our total DELTA cohort. Homocysteine (tHcy) levels were measured together with saturated fatty acids (FAs), monounsaturated fatty acids (MUFAs) and polyunsaturated FAs (PUFAs) of the omega (N)-3, omega-6 and omega-9 series in plasma and erythrocytes. Compared to laboratory reference values our study sample showed lower levels in erythrocytes of C22: n-3, C22: 6 n-3, C24: 1 n-9 and C20: 3 n-9 and in plasma a decrease in C24: 1 n-9 and C20: 3 n-9. These differences were only statistically significant for the total of n-6 fatty acids and plasma tHcy. The fact that most patients in our sample were clinically recovered suggests that the FA alterations may represent a biological “trait” marker for MDD-R.

In chapter 4.2 (research question 4 a and b) we confirmed the results of chapter 4.1. using the results of a case-control study with 137 patients with MDD-R and 65 matched non-depressed controls. This study confirmed the results of our explorative pilot study. The most striking finding is that in both plasma and erythrocytes of patients with MDD-R the concentrations of most of the SFAs and MUFAs, and additionally erythrocyte PUFAs, all with a chain length of 20 carbon (C) atoms, were significantly lower than in the controls. In contrast, the concentrations of most of the shorter chain members (18C) of the SFAs and MUFAs were significantly higher in patients. Estimated activities of several elongases in plasma of patients were significantly altered, whereas delta-9 desaturase activity for C14:0 and C18:0 was significantly higher.

The fatty acid status of patients with MDD-R not only differed with regard to omega-3 and omega-6 PUFAs, but this was also the case for other fatty acids. The alterations observed may be
due to differences in diet, changes in synthesizing enzyme activities or higher levels of chronic (oxidative) stress. However, these alterations may also be the result of adaptive strategies by providing protection against enhanced oxidative stress and production of free radicals.

In chapter 4.3 (research question 5) we investigated whether the Thr54-polymorphism in the \( FABP2 \)-gene: (I) was more prevalent in MDD-R patients than in sex-and age-matched controls, (II) was associated with observed alterations in FA-metabolism, and (III) was associated with the CVD-risk factor waist circumference. \( FABP2 \)-genotype distribution did not significantly differ between the 137 MDD-patients and 73 matched controls. However, patients with the Ala54Thr-polymorphism had higher concentrations of especially eicosadienoic acid and other 20-carbon FAs, and was associated with lower waist circumference. In addition, \( FABP2 \)-genotype effects on waist circumference in patients seemed mediated by its effect on C20:2 n-6, and different from controls.

Although Ala54Thr-polymorphism distribution was not associated with MDD-R, our results indicated that \( FABP2 \) may play a role in the explanation of observed FA-alterations in MDD. For patients with the Ala54Thr-polymorphism, potentially adaptive conversion of increased bio-available dietary precursors into eicosadienoic acid instead of arachidonic acid might be related to a low waist circumference.

One Carbon metabolism

In chapter 5 (research question 6), we studied the genetic variation in the enzymes of the one-carbon (1-C)-metabolism and integratively investigated key 1-C-components (folate, homocysteine, vitamin \( B_6 \) and \( B_{12} \)), while including the possible effects of antidepressant medication and depressive state to gain more insight in the possible association between the MTHF-polymorphism and MDD-R. We compared the \( MTHFR \) C677T-polymorphism together with the key 1-C-components in clinically ascertained patients with MDD-R (n=137) to age- and gender-matched healthy controls (n=73). Despite our specific recurrently depressed patient population, we found no clear associations with the 1-C-cycle, except for higher homocysteine and lower vitamin \( B_6 \) during the depressed state. This suggests that 1-C-cycle alterations in MDD-R are state-dependent, possibly resulting from high levels of acute (psychological) stress. Stress reduction may be a treatment target to lower cardiovascular risk in this population.

In chapter 5 (research question 7) we also showed that the relation between the C677T \( MTHFR \) variant and depression recurrence, over a 5.5-year follow-up, was modified by previous
exposure to traumatic childhood events (TCEs) in a clinical sample of 124 patients with MDD-R. This finding was supported by the fact that in an independent replication sample of 665 healthy individuals from the general population the relation between the C677T MTHFR variant and self-reported depressive symptoms was also modified by TCEs. In summary: T-allele carriers exposed to childhood trauma may be at increased risk for depressive symptoms or MDD recurrence.

Medically unexplained physical symptoms
In chapter 6 (research question 8), we show that medically unexplained physical symptoms (MUPS) are a risk factor for recurrence in recurrent depression over 3.5 years. The relation between MUPS and recurrence of depression could not be explained by an association between sustained high level of MUPS and omega (ω)-3 and −6 fatty acid (FA)-status and/or functional polymorphisms in the promoter region of the serotonin transporter gene (5-HTTLP). We conclude that recognition and reduction of MUPS within this subsample of patients in an early state could prevent a (depressive) relapse.

General discussion
MDD is a heterogeneous disorder with a highly variable and recurrent course, an inconsistent response to treatment, and so far no established (biological) mechanism. When taking into account the recurrences of MDD, it is a challenge to understand dynamic changes in psychobiological systems over time and investigating different biological systems in the course of MDD. During the time-period in which the studies that contributed to this thesis were conducted, many manuscripts in the field of metabolic and biological factors in the course of MDD have been published. This stipulates the importance of stress-related factors in particular, which has led to a growing awareness that this topic may be considered as an important research and clinical focus.

Epidemiologic findings point to an association between MDD and increased cardiovascular morbidity and mortality. In many patients, cardiovascular disorders precede depression, but in others, depression precedes the cardiovascular disorder. Both n−3 fatty acid deficiency and elevated plasma homocysteine levels have been implicated in cardiovascular disease (CVD) and in MDD. Elevated cortisol levels in depression might increase the risk of CVD, given that cortisol increases visceral fat.

So, among the pathophysiological systems that may play a role in the etiology of MDD and its recurrent nature are the hypothalamic-pituitary-adrenal (HPA) axis and some metabolic abnor-
malities. HPA-axis hyperactivity has been demonstrated in depressed persons compared with controls, and has been further implicated as a potential mechanism through which depression increases the risk of CVD and other somatic diseases. Related to this, other metabolic abnormalities such as obesity and adverse lipoprotein patterns are also associated with MDD and several studies reported an association between MDD and the metabolic syndrome, a cluster of risk factors for type 2 diabetes mellitus and CVD.

In this thesis, we simultaneously assessed these biological factors (HPA-axis functioning, fatty acid metabolism and the 1-C cycle components) in a population with recurrent MDD. We considered two hypotheses presented in the introduction to explain the high risk for recurrence: (I) the vulnerability-accumulation or scarring hypothesis and (II) the premorbid vulnerability hypothesis. We did so by investigating premorbid factors (e.g. genetics, childhood trauma), which are present before MDD onset, as well as biological factors that can play a premorbid role and/or are involved in vulnerability-accumulation (‘scarring’).

Although the results of our studies could not yield a clear direction to either one of the two hypotheses, they both showed an effect of premorbid and scarring factors in the course of recurrence in our MDD patients. In addition, we found some evidence for a premorbid (genetic) vulnerability in the FA and 1-C metabolism, suggesting a premorbid vulnerability for CVD-risk and MDD. Furthermore, we found (biological) stress vulnerability factors, e.g. a persistent trait of increased cortisol concentrations and 1-C alterations. These metabolic alterations, possibly in combination with a changed lifestyle, were associated with a worse course of MDD-R. When integrating the findings concerning these pathophysiological mechanisms (HPA-axis, FA and 1-C metabolism), a common pattern of specific biological alterations emerges in MDD-R patients. In this discussion we choose to zoom in on two specific topics, namely the concept of oxidative stress and the effects of childhood trauma.

Oxidative stress
We argue that oxidative stress - defined as an imbalance between production and inactivation of reactive oxygen species (ROS) - may play a fundamental role in the pathogenesis of both MDD and CVD, and so may function as their common denominator. Oxidative stress is associated with alterations in the intrinsically linked FA-metabolism and 1-C-cycle. Both are essential for adequate neurocognitive functioning and mood regulation, as well as a proper functioning of the cardiovascular system.
Altered FA-metabolism has been consistently reported in MDD\textsuperscript{11,12,13}, both in acutely depressed and remitted patients\textsuperscript{11}. The main findings, also in our studies, are lower concentrations of n-3 long chain polyunsaturated fatty acids (LCPUFA) [e.g. eicosapentaenoic acid (C20:5 n-3; \textit{EPA}) and docosahexaenoic acid (C20:6 n-3; \textit{DHA})\textsuperscript{11,12}], and decreased overall FA unsaturation, chain length and peroxidizability\textsuperscript{14}. FAs together with their (non)enzymatic peroxidation products may very well explain (part of) the overlap of the clinical picture between CVD-risk factors and MDD.

To understand the effects of oxidative stress on FA-metabolism, we suggest that the 1-C-cycle plays an essential integrating role in modulating the effects of oxidative stress on FA-metabolism. The 1-C-cycle shifts away from the methylation pathway and production of methyl groups needed for PUFA-production, neurotransmitters, and DNA-methylation, to the transsulfuration-pathway resulting in synthesis of the major intracellular antioxidant glutathione.

Increased oxidative stress may be intrinsically involved in the shared disposition for both MDD and CVD. Increasingly, evidence indicates that disturbances of the antioxidant defense system and presence of oxidative stress may play a role in the biochemical mechanisms underlying psychiatric disorders, e.g. MDD\textsuperscript{15,16}. It is likely that the proposed mechanism also occurs at the level of the neuronal membrane, which is the site of neurotransmitter receptors, ion channels, signal transduction, and drug effects. Biological systems have evolved complex protective strategies against free radical toxicity. Under physiological conditions the potential for free radical-mediated damage is kept in check by the antioxidant defense system, which is comprised of a series of enzymatic and non-enzymatic components\textsuperscript{16}.

In short, oxidative stress is a state of dysequilibrium between pro-oxidant processes and the antioxidant defense system in favor of the former. Since the role for toxic radicals in the etiology of schizophrenia was proposed in the 50s\textsuperscript{17}, there are several studies linking free radical mechanisms to the pathophysiology of psychiatric disorders.

Recently, MDD was shown to be related to lower plasma concentrations of several key antioxidants, such as vitamin E, zinc and coenzyme Q10, as well as lower antioxidant enzyme activity\textsuperscript{18}. These deficiencies in antioxidant defenses impair protection against reactive oxygen species (ROS), and lead to damage of fatty acids, proteins and DNA. Moreover, there is an association between depression and polymorphisms in genes involved in oxidative pathways, affecting enzymatic activity\textsuperscript{18}. Taken together, this suggest a role of free radicals and antioxidants in the pathophysiology of (recurrent) MDD.
Both heritable genetic factors and environmental factors including dietary fatty-acid composition may act in concert to sustain elevated immune-inflammatory signaling. Converging translational evidence has shown elevated immune-inflammatory signaling activity in the pathophysiology of mood disorders, including MDD\textsuperscript{19,20}, and even suggesting different MDD-subtypes (e.g. inflammatory and metabolic dysregulation associated with atypical depression).

**Long term effects of Childhood Trauma**

Our data suggest that exposure to traumatic events during childhood (TCE) influences or changes the regulation of the HPA-axis. We found an indication that childhood trauma slightly confounded the prediction of recurrence by mean morning cortisol. In our other study, we found that CLEs, not specified as trauma, did not explain the observed hypercortisolemic trait. Finally, our study showed that MDD patients with a childhood trauma history and carriers of the thermolabile variant of the \textit{MTHFR} gene constitute a subgroup of patients that had a worse course of recurrence. These biological alterations may in turn be a vulnerability factor for the onset of MDD-R itself. Our findings indicate that childhood trauma contributes to the risk on recurrences of MDD. The effect of environmental factors on the course of MDD, and childhood trauma in particular, is in line with a recent meta-analysis\textsuperscript{21} and a study by Peyrot et al.\textsuperscript{22}.

The experience of traumatic events in childhood may have a crucial role on the HPA-axis, in dealing with stress, and subsequently, in onset and course of depression (chronicity). Childhood traumas have been reported before as an independent determinant of chronicity of depression\textsuperscript{23}. This is in line with our finding that lower cortisol levels within this patient group were associated with having experienced more traumatic life events in childhood. Lower cortisol levels were also predictive of prospective recurrence (42.3% of the patients with lower cortisol experienced traumatic life events while 19.2% experienced childhood trauma in patients with higher cortisol). Treadway et al.\textsuperscript{24} suggest that chronic stress subsequent to childhood maltreatment may initiate glucocorticoid-related injury to the anterior cingulate cortex. This damage may impair cortico-limbic circuits involved in emotion regulation.

Carpenter and colleagues reported that especially emotional childhood abuse might dampen cortisol reactivity and that this effect is cumulative overtime\textsuperscript{25}, as was shown in a study amongst 230 adults without major Axis I Disorders that completed the Dex/CRH test. Unfortunately, we do not have data on emotional abuse in childhood to examine this hypothesis. However, we did indeed find an indication that childhood trauma in general (such as sexual abuse) slightly confounded the prediction of recurrence by mean morning cortisol. Our finding that CLEs, not
specified as trauma, did not explain the observed hypercortisolemic trait, is in line with previous literature 26.

Childhood trauma may be conceptualized as a developmental risk factor triggering a chain of risks such as subsequent depressive episodes that might progressively potentiate the vulnerability to poor course of illness 27. In order to understand the origins of this chain of risks, future studies should explore the cognitive and biological correlates of trauma in childhood before the accumulation of MDEs 28.

Furthermore, it is important to characterize the gene-environment interplay underlying the effects of childhood trauma on MDD outcomes. Childhood trauma may be conceptualized as an environmental risk factor for poor MDD course and a moderator of treatment outcome, complementing the emerging genetic markers of vulnerability to recurrent depression 29 and poor treatment response 30.

The experience of a traumatic event during childhood may disrupt major beliefs regarding personal invulnerability, benevolence of the world, meaning, self-worth, and relations with others. An individual may feel vigilant, depressed, powerless, vulnerable or guilty about not being able to change the situation, and these feelings may color the way the individual sees the world. This may increase vulnerability for psychiatric disorders such as MDD. The association of childhood trauma with MDD in adulthood could be due to common factors linking family perpetrators of abuse and their victims, including not only shared genes but also a shared environment (e.g. poverty, poor nutrition and poor prenatal care) 31. Moreover, Nederoff & Schmidt 32 propose that individual differences in sensitivity to early programming in combination with the environments encountered during sensitive periods determine whether the cumulative stress hypothesis (disease risk increases as adversity accumulates) or the mismatch hypothesis (a stressful childhood leads to developmental changes to prepare for a stressful adulthood) is more applicable.

Clinical relevance
What are the consequences of our findings for clinical practice? Knowledge of specific predictors and underlying mechanism(s) of MDD recurrences is essential for making treatment decisions. About half of the patients with MDD in mental health care have recurrences and the risk increases with higher (HRSD>10) inter-episodic symptomatology 33. It is important to closely monitor these high-risk patients for relapse, for instance by using mobile devices that are users friendly for daily life (such as mobile phones, Apps 34).
Long-term use of antidepressants is recommended for patients that remitted with antidepressants by leading international guidelines for recurrently depressed patients \(^{35, 36, 37}\). However, a recent meta-analysis demonstrated that with an increasing number of episodes a relative resistance against the protective effect of antidepressant medication is developed \(^{38}\).

Several meta-analyses demonstrated that psychological interventions applied in the acute phase of the depressive episode, have an enduring effect after remission \(^{39, 40}\). In addition, rather than a relative resistance with increasing numbers of episodes, an increasing protective effect is found for psychological interventions, including MBCT \(^{41, 42, 43, 44}\).

Recent studies indicate that psychological preventive interventions might be an alternative strategy for long-term use of antidepressants for this recurrent patient group as first studies already indicate \(^{42, 43}\). However, further large-scale studies are needed to validate this positive effect of psychological interventions as an alternative for antidepressant maintenance treatment.

A sequential approach in which a brief psychological intervention (i.e. Preventive Cognitive Therapy, Maintenance Cognitive Therapy, and Mindfulness based Cognitive therapy) is started after recovery on other treatment (including AD treatment) is effective in preventing recurrence as demonstrated by several meta-analyses, especially for patients with MDD-R (for meta-analyses see \(^{40, 41, 45, 46}\)).

Presently, relapse rates are still (too) high. To reduce recurrence rates in MDD, a personalized medicine approach using specific markers, including clinical, genetic, biological and psychological markers, might help us to examine which preventive strategy works for whom at what stage \(^{47}\). A potential target for relapse prevention is the ability to better handle or cope with recurrent stressors and daily hassles.

Additionally, development and evaluation of new interventions specifically focused on the subsample of these patients with MUPS might also improve outcome over time. Especially an intervention that focuses on the ability to notice, but not over-react to MUPS and its negative bodily sensations and experiences, might be promising. More empirical evidence in support of these potential strategies is needed, however, before firm conclusions may be drawn.

The larger part of the variance in recurrence remains unexplained. This makes more and prospective research needed to better identify (biological) predictors play a role in recurrences.
It could also be useful to apply different outcome variables (severity, time between recurrences, number of recurrences) because different factors could predict different outcomes. Moreover, as a result, recurrence of MDD remains difficult to predict and stays a complex and only partially understood phenomenon.

Treatment of the high-risk MDD-R group should aim for the reduction of biopsychosocial risk factors for recurrence. This approach has been successful in other disciplines in medicine. For example, in our intention to treat analysis, CT had an effect of steeper cortisol declines over the day throughout the 2-year follow-up. The mechanisms underlying the observed effect of CT on the HPA-axis are not elucidated yet. It could be hypothesized that the preventive CT changes coping strategies, e.g. stress perception, management of stress and generation of subsequent stress. These effects could mediate its recurrence-preventive effects. As this evidence accumulates it may be possible to design more effective and efficient models of relapse prevention, to reduce the burden that MDD-R places on those who are affected.

MDD-R patients with early trauma and specific psychotherapies
This section focuses on two, in our opinion, important topics of clinical relevance; (I) patients that premorbid experienced factor Childhood Trauma and (II) interventions targeting the 1-C cycle and thereby lowering CVD-risk.

A meta-analysis including epidemiological studies demonstrates that maltreated individuals were twice as likely as those without a history of childhood maltreatment to develop both recurrent and persistent depressive episodes. The results from clinical trials corroborated these epidemiological observations. Further, compared with depressed individuals without a history of childhood maltreatment, depressed and maltreated individuals appeared to benefit less from treatment (and particularly from combined treatment), thereby incurring greater risk of recurrent and persistent depressive episodes.

Our results suggest a subgroup of patients for whom interventions should focus on both MDD recurrence and the consequences of childhood trauma. The gene-environment interaction we found in two independent samples suggests benefit from the integration of two types of therapeutic approaches. Psychotherapeutic interventions specifically aimed at the consequences of childhood trauma while there is no PTSD (including psychotherapeutic treatment of trauma related problems), which could be combined with interventions aimed at the 1-C cycle.
Information about a history of childhood trauma and maltreatment helps to identify individuals who are at high risk of developing a recurrent and persistent subtype of depression and those who will respond poorly to current treatments. Clinicians may consider that the routine inquiry about childhood maltreatment is not harmful and could add important prognostic information to their assessment. Clinicians may also consider more intensive and alternative treatment options for recurrent MDD individuals with a history of childhood trauma.

The meta-analytical evidence that maltreated and depressed individuals have a poor response to combined treatment with structured psychological therapy and antidepressant medications indicates that simply combining these two common options is not sufficient. It is important to consider exploring the response of maltreated and depressed individuals to new treatments targeting the biological vulnerabilities described in this subgroup.

Interventions aimed at reducing childhood maltreatment could help to reduce the large health and economic burden linked to poor MDD course. Childhood years are thought to be a sensitive developmental window for the maturation of emotion regulation. Therefore, early preventive and therapeutic interventions (even in childhood or adolescence) may be more effective (and cost-effective) in preventing a poor longitudinal recurrent course of MDD than interventions at later ages, when harmful developmental trajectories have already been established.

Interventions aimed at the 1-C cycle, and lowering CVD-risk

In patients with recurrent MDD, their cardiometabolic risk factors and MetS status should be carefully monitored, and proper treatment and lifestyle changes could be advised if the patients are at a higher risk of diabetes/CVD.

(Oxidative) stress may be mitigating by improving anti-oxidant defenses through dietary modification and (add-on) exercise. MDD is commonly associated with lower levels of physical activity. While data derived from epidemiological and correlational studies do not necessarily confirm causation, a consistent relationship does exist across a number of populations. In adults, an active lifestyle was associated with reduced depressive symptoms independent of education and physical health status. In overweight/obese adults, a reduced risk of MDD was associated with increasing moderate-to-vigorous-intensity physical activity and decreasing sedentary time. Another study using data from over 4,000 men and women aged 20 years or older confirmed that adults with depression spent significantly less time both in light and moderate physical activity than non-depressed adults. In a longitudinal study of over
9000 people, regular physical activity was associated with a reduced likelihood of depressive symptoms at follow-up 55.

The efficacy of exercise as a treatment for MDD is summarized in a lot of recent reviews. In a meta-analysis on supervised and unsupervised physical activity interventions among healthy adults, Conn 56 concluded that physical activity interventions had a moderate inhibitory effect on depressive symptoms in adults with and without clinical depression. Carek et al. 57 concluded from a review of the literature that exercise compared favorably to AD medications as a first-line treatment for mild-to-moderate depression and also improved depressive symptoms when used as an adjunct to medications. Similar antidepressant effects were also found in trials comparing exercise with cognitive-behavioral therapy 58. Despite these positive findings there is a paucity of research demonstrating long-term beneficial effects of exercise in patients with clinical MDD 59, 60.

Prevention and/or treatment of CVD and MDD-R should primary combat underlying oxidative stress. Therefore, we propose an effective approach, lowering oxidative stress by physical exercise, a healthy diet, reducing psychological stress (e.g. cognitive therapy and/or antidepressants), and weight loss. All have been proven beneficial for psychiatric symptomatology and CVD-risk 61, 62. These interventions should be routinely implemented in clinical care for MDD-R patients.

Moreover, (adjuvant) novel oxidative stress relieving treatments seem promising. For example, effects on oxidative stress of N-acetylcysteine through the 1-C-Cycle, but also psychotherapy 62, 63, may be an interesting focuses of future investigation.

Finally, in spite of consensus recommendations and guidelines, proper monitoring of anthropometric and metabolic parameters has not yet been strictly implemented in psychiatric care. Obstacles to implementation need to be overcome by making CVD-risk monitoring mandatory. The concept metabolic syndrome (MetS) encompasses a cluster of CVD-risk factors and may be a helpful tool for clinicians to assess CVD-risk. Although there is continuing debate regarding the MetS-criteria and -concept, this clustering of risk factors is unequivocally linked to an increased risk for developing CVD 64, 9. The concept MetS could help clinicians which recurrent MDD patients that should receive treatment for their increased CVD-risk 10.
Methodological considerations

Limitations

Limitations of the studies in this thesis were discussed in each chapter. However, we will summarize the most important ones in the next paragraph.

First: Our study sample is comprised only of patients with recurrent depression. It is not clear whether our results will generalize to patients with MDD in general. Restricting ourselves to MDD-R patients, however, had as a benefit that this subgroup represents a more biologically determined MDD-subtype, which may be specifically linked to recurrence and CVD-risk. For this reason, we expect these patients to deviate strongest from healthy controls in the (patho) physiological and genetic mechanisms and to have more pronounced alterations in their course of MDD which is the main focus of our thesis.

Second: We applied a prospective cohort approach in some of the studies used in this thesis to the data of patients who originally participated in a randomized controlled preventive cognitive therapy (PCT)-trial. Where possible, the analyses were performed on the total sample, with and without instant drop-outs (PCT plus treatment as usual (TAU), n=172; early drop-outs immediately after inclusion, n=15). To account for the fact that our sample participated in a RCT, we assessed whether the intervention (PCT) modified or the relation between potential risk factors and the outcomes and if this was the case performed the analyses only in the control group (n=84), it is therefore unlikely that the intervention has biased our results.

For some analyses we only included those patients (a) who attended the study-protocol specific follow-up moments and had a valid research variable (e.g. PSC-questionnaire, cortisol, 1-C-components) and who were euthymic (had no DSM IV-R diagnosis of major depressive episode) in the observed period. In these cases, we always tested whether the study-protocol specific included subgroups of patients was comparable to the other (excluded) patients on demographic and clinical variables.

Third: The issue of multiple imputation in some studies of this thesis. Although we used multiple imputation to reduce bias introduced by missing values, it is possible that the missing values influenced our result. Multiple imputation is based upon the assumption that data are missing at random. This means that missingness (i.e. whether data are missing or not) may depend on observed data, but not on unobserved data. In our studies, we were able to include several predictors in the imputation model (e.g. psychopathological, demographic, and other biological
variables), which increases the chance that missingness is accounted for by observed data. In addition, most missing data in our studies is missing completely at random, e.g. due to laboratory or logistic accidents, which in any case would not result in biases. Furthermore, there is some evidence that even in the case that missing is not completely at random, analyses using multiple imputation will yield less biased results than analysis using other methods like for instance complete cases analysis. Furthermore, bias by missing values would not easily explain the observed relations. Comparing results with and without multiple imputations should be done with caution. However, replication of our findings would strengthen the evidence.

Fourth: One could question whether our patient sample is a representative (sub) group of high-risk recurrent MDD patients. This may not be completely the case. The patients in the studies described in this thesis, although remitted, were willing to participate in a preventive CT trial and therefore are self-selective.

Fifth: We assessed childhood trauma with a self-report questionnaire rather than an interview. This might on the one hand underestimate the actual prevalence of childhood trauma, or on the other hand due to social desirability lead to over-estimate.

Strengths
First, a major strength of this thesis is that all the research aims were investigated within in a specific sample of highly recurrent depressed patients, which can be considered characteristic for those patients particularly representative of a more biologically determined MDD-subtype, which may be specifically linked to recurrence and cardiovascular co-morbidity. Therefore, the included patient group can be considered characteristic for those patients particularly causing the large MDD-associated burden of disease. Moreover, these patients give the biggest contrast. Where there are biological differences to find than they are most likely to be found in this group.

Second, by using a prospective design it was possible to study the predictive value of various potential biological risk factors for the recurrent course of MDD during a follow-period (maximum of 5.5 years).

Third, it is rather unique to study biological, psychological, cognitive and somatic processes in a comprehensive battery ranging from self-reported measures, interview based measures to biological and genetic tests in such a well-defined clinical population.
Future perspectives

Based on the findings and conclusions from the studies that were presented in this dissertation, we suggest that it should be considered good practice to include healthy controls in studies examining recurrent MDD, HPA-axis regulation, and metabolic and genetic mechanisms and thereby following both groups prospectively. In studies examining the relationship between trauma exposure and polymorphisms and/or metabolites, adequate trauma assessment, not only of the patients but also of the controls should take place. This trauma assessment should not only cover trauma exposure in adulthood but more importantly, also trauma exposure during childhood.

Finally, it would be very intriguing to compare healthy controls, individuals before onset of MDE, patients with one MDE and the recurrent MDD patients on the biological factors longitudinal investigated in this thesis. Especially, examining biological factors (in combination with environmental factors) over time in these groups, may resolve the issue that for some patients MDD is an acute time-limited condition, while for others it results in recurrent episodes over the life course. Currently, there is little information available to guide researchers and clinicians in understanding which of these two life course pathways any particular person with a first MDE eventually will follow.
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Appendices
Appendices

Nederlandse samenvatting en conclusies
Samenvatting
Het oorspronkelijke doel van dit proefschrift was om de kennis te vergroten over de mogelijke biologische factoren, het psychopathologische mechanisme (inbegrepen de werking van de HPA-as, one-carbon en vetzuur metabolisme), en de gen-omgevingsinteracties voor het beloop van recidiverende depressie (Recurrent Major Depressive Disorder MDD-R). Het bestuderen van MDD-R is belangrijk, omdat er aanwijzingen zijn dat deze een meer biologisch en genetisch vastgesteld MDD-subtype vertegenwoordigt, welke gerelateerd zou zijn aan terugval en de kans op cardiovasculaire aandoeningen (CVD). Dit is de reden waarom wij verwachtten dat deze patiënten het sterkst zouden afwijken van gezonde controlepersonen in de (patho) psychologische en genetische mechanismen en het hebben van meer uitgesproken veranderingen in hun beloop van MDD.

Twee leidende hypotheses geven verklaringen voor het onderliggende (kwetsbaarheids-) mechanisme voor de verhoogde kans op recidivering: (I) de 'vulnerability-accumulation' of 'scarring' hypothese en (II) de premorbide 'vulnerability' hypothese. Ons doel was om specifieke premorbide factoren te onderzoeken (zoals genetica en traumatische ervaring in de jeugd) die aanwezig zijn voordat de eerste episode van MDD optreedt, als ook de biologische factoren die een premorbide rol kunnen spelen en/of factoren die wijzen op vulnerability-accumulation ('scarring'). Deze biologische variabelen zijn verzameld in verschillende stadia van het beloop van MDD-R, zoals in de herstellende fase, subsyndromale fase en in de acute depressieve episode.

Studies in deze dissertatie zijn voornamelijk gebaseerd op gegevens die verzameld zijn bij deelnemers aan de DELTA studie: een goed gedefinieerde populatie met een hoog risico voor terugval en recidivering, omdat het exclusief herstelde patiënten betreft met tenminste twee voorafgaande depressieve episodes.
Onderzoeksvragen

Deze dissertatie bestaat uit deelstudies die de volgende vragen die betrekking hebben op pathofysiologische mechanismen bij het beloop van MDD-R, willen beantwoorden:

1  Wat is de relatie tussen terugkerende depressie, overgewicht en obesitas bij patiënten met MDD-R en wordt deze relatie gemodificeerd door het gebruik van antidepressieve (AD) medicatie?

2a  Op welke wijze verschilt de werking van de HPA-as bij patiënten met MDD-R en gezonde controlepersonen?

2b  Heeft dit een blijvend karakter (“trait”) of wordt dit beïnvloed door de depressieve episode (“state”), dan wel stress of vorige episodes, en is dit geassocieerd met terugval?

2c  Kan deze relatie gemoduleerd worden door cognitieve therapie?

3  Voorspellen HPA-as metingen de tijd tot terugval bij MDD-R patiënten?

4a  Verschillen niveaus van meervoudig onverzadigde vetzuren (PUFA) en de n-6/ n-3 ratio tussen patiënten met MDD-R en niet depressieve controlepersonen?

4b  Zijn deze mogelijke veranderingen “state” afhankelijk, of geven deze de huidige depressieve “trait” weer (t.w. zijn ze onafhankelijk van de huidige depressieve episode)?

5  Wat is de relatie van het Ala54Thr vetzuur proteïne-2 (Fatty Acid Binding Protein (FABP2)) polymorfisme in MDD-R en zijn vetzuur concentraties gerelateerd aan CVD- risico (bepaald als de risicofactor taille-omtrek)?

6  Wat is de invloed van het MTHFR polymorfisme en de relatie ervan met de 1-Carbon-cycle (1-C-cycle) componenten bij patiënten met MDD-R, vergeleken met controlepersonen en worden deze beïnvloed door de depressieve “state” en/of het gebruik van AD?

7  Wat is de relatie tussen MDD-R patiënten die een T-allel in the MTHFR gen dragen met tijd tot terugval en is dit gerelateerd aan blootstelling aan traumatische ervaringen in de kindertijd (TCE)?

8  Wat is de associatie tussen niet verklaarde medische fysieke symptomen (Medically Unexplained Medically Symptoms (MUPS)) en tijd tot terugval in depressie en wat is de associatie tussen MUPS en het vetzuurmetabolisme en MUPS en het serotonine transporter?
Samenvatting van de resultaten van de studies

Obesitas

In hoofdstuk 2 (onderzoeksvraag 1) lieten we zien dat overgewicht en obesitas meer voorkwam bij patiënten met MDD-R dan bij de referentie groep. Dit was enkel statistisch significant bij vrouwen (74% van de proefpersonen). MDD-R patiënten die AD gebruikten (voornamelijk SSRIs) hadden beduidend meer obesitas dan degenen die ADs tijdelijk of helemaal niet gebruikten. Vergeleken bij SSRIs had het gebruik van andere type ADs (t.w. tricyclisch ADs) geen impact op de antropometrische metingen. Echter, we hebben een kleine associatie gevonden tussen de AD equivalentdosering en de omtrek van de taille en de ratio van taille tot heup.

Deze studie is gedaan bij MDD-R patiënten ($n=134$) en is vergeleken met de algemene bevolking (project MORGEN), wat betreft BMI en de omtrek van de taille.

Een beter begrip van de relatie tussen obesitas en depressie en de nuttige en ongunstige effecten van psychotropics op eetlust, eetgewoonte, gewicht en metabolisme, zou het voorkomen en behandelen van zowel obesitas als depressie kunnen verbeteren. Dit steunt de ontwikkeling van op de persoon geijkte interventies voor MDD-R, inclusief effectieve non-farmaceutische preventieve behandelingen en extra fysieke activiteiten welke - als toegevoegd voordeel - bescherming zou kunnen bieden tegen gewichtstoename teweeggebracht door AD.

HPA-as

In hoofdstuk 3 (onderzoeksvraag 2), beschrijven we dat patiënten met MDD-R een hogere concentratie cortisol hadden dan gender en leeftijd gematchte controlepersonen. Dit veranderde niet gedurende nieuwe MDD-episodes die optraden gedurende de follow up (3 maanden en 2 jaar). HPA-as activiteit was niet gerelateerd aan dagelijkse beslommeringen of life-events in de kinderjaren. Interessant was dat de concentraties cortisol lager waren bij patiënten die meer eerdere episodes hadden, maar niet geassocieerd waren met terugval(len) gedurende de tijd dat wij de patiënten prospectief volgden. Uiteindelijk bleek dat random toegewezen cognitieve therapie bij de start van het onderzoek cortisol daling over de dag versterkte gedurende de 2-jaar follow-up.

Deze resultaten geven aan dat herstellende MDD-R patiënten aanhoudend verhoogde cortisol concentraties hebben, ongeacht stress. In combinatie met de bevindingen dat concentratie van cortisol bij patiënten niet verandert gedurende nieuwe MDD-episodes (en dus niet wijzen op ‘epiphenomenal’ of stemmingseffecten), ondersteunen onze resultaten de conclusie dat hypercortisolemie voldoet aan het criterium voor een endofenotype van MDD-R.
Ook richten we ons in dit hoofdstuk (onderzoeks vraag 3) op de observatie, dat gemiddeld lagere ochtendwaarden van cortisol een kortere tijd tot terugval over 5.5 jaar voorspelden binnen deze groep na correctie van residuele symptomen. Residuele symptomen en trauma uit de kindertijd waren confounders van de associatie tussen cortisol en terugval. Lagere cortisol niveaus werden geassocieerd met traumatische ervaring tijdens de jeugd (42,3% van patiënten met lager cortisol had een dergelijke ervaring versus 19.2% bij patiënten met hoger cortisol).

Deze studie ondersteunt de voorspellende rol voor terugval over 5.5 jaar van een gedysreguleerde HPA-as (t.w. lagere ochtend cortisol niveaus) bij patiënten met recidiverende depressie. Jeugdtrauma werd geassocieerd met het hebben van lage cortisol niveaus. Dit zou op termijn consequenties kunnen hebben om met (psychologische) stress om te gaan vergezeld van deze HPA-as veranderingen.

Vetzuur metabolisme

Hoofdstuk 4.1 (onderzoeks vraag 4.a) beschrijft eerst een exploratieve studie waarin we random patiënten hebben geselecteerd uit het DELTA cohort.

Homocysteine (tHcy) niveaus werden gemeten samen met verzadigde vetzuren (FAs), enkelvoudige onverzadigde vetzuren (MUFAs) en meervoudig onverzadigde vetzuren (PUFAs) en omega (N)-3, omega-6 en omega-9 series in plasma en erytrocyten. vergeleken met laboratorium referentiewaarden liet onze studie lagere waarden zien in erytrocyten van C22: n-3, C22: 6 n-3, C24: 1 n-9 en C20: 3 n-9 en in plasma een daling in C24: 1 n-9 en C20: 3 n-9. Deze verschillen waren alleen statistisch significant voor het totaal van n-6 vetzuren en tHcy. Het feit dat de meeste patiënten in ons sample klinisch hersteld waren, suggereert dat de FA veranderingen een biologische “trait” marker zouden kunnen representeren voor MDD-R.

In hoofdstuk 4.2 (onderzoeks vraag 4. a) en b) bevestigden wij de uitkomsten van hoofdstuk 4.1, waarbij we de resultaten van een case-control studie bij van137 patiënten met MDD-R en 65 niet-depressieve controlepersonen hebben gebruikt. Deze studie bevestigde de resultaten van onze pilot studie. De meest opvallende ontdekking is dat in zowel plasma als in erythrocyten van patiënten met MDD-R de concentraties van de meeste van de SFAs en MUFAs, en additieel erytrocyt PUFAs, allen met een reeks lengte van 20 carbon (C) atomen, beduidend lager waren dan in de controles. Het tegenovergestelde was het geval bij de concentraties van de meeste kortere reeks leden (18C) van de SFAs en MUFAs. Deze waren beduidend hoger bij MDD-R, patiënten. De geschatte activiteit van verschillende elongases in het plasma van patiënten waren
beduidend gewijzigd, terwijl de delta-9 niet verzadigde activiteit voor C14:0 en C18:0 beduidend hoger was.

De vetzuurstatus van patiënten met MDD-Rverschilden niet alleen met betrekking tot omega-3 en omega-6 PUFAs, maar ook in andere vetzuren. De geobserveerde veranderingen zouden toe te schrijven kunnen zijn aan verschillen in dieet, veranderingen in de synthese van de enzymwerking, of een hoger niveau van chronisch (oxidatieve) stress. Echter, deze wijzigingen kunnen ook het resultaat zijn van adaptieve strategieën door bescherming te bieden aan verhoogde oxidatieve stress en de productie van vrije radicalen.

In hoofdstuk 4.3 (onderzoeksvraag 5) onderzochten wij of het Thr54-polymorfisme in het FABP2-gen (I) meer prevalent was bij MDD-R patiënten dan in gender en leeftijd gerelateerde controlepersonen, (II) geassocieerd was met geconstateerde veranderingen in het vetzuurmetabolisme, en (III) geassocieerd was met de CVD risicofactor, namelijk de omtrek van de taille. De distributie van het FABP2-genotype verschilde niet tussen de 137 MDD-patiënten en 73 controlepersonen. Echter, patiënten met het Ala54Thr-polymorfisme hadden een hogere concentratie van voor-namelijk het eicosadienoic vetzuur en andere 20-carbon FAs, en werd gerelateerd met een smallere taille omtrek. Bovendien, FABP2-genotype effecten op de taille omtrek bij patiënten bleek gemedieerd te worden door het effect op C20:2 n-6, en dit verschilde van controles.

Hoewel de distributie van Ala54Thr-polymorfisme niet geassocieerd was met MDD-R, geven onze resultaten een indicatie dat FABP2 een rol zou kunnen spelen in de verklaring van geobserveerde vetzuur-wijzigingen. Bij patiënten met het Ala54Thr-polymorfisme zijn mogelijk potentiële adaptieve conversies van verhoogd biobeschikbare dieet precursoren, van eicosadienoic zuur in plaats van arachideolie zuur, gerelateerd aan een smallere taille omtrek.

Een Carbon metabolisme

In hoofdstuk 5 (onderzoeksvraag 6) hebben we de genetische variatie van de enzymen van het 1-carbon (1-C)-metabolisme in combinatie met de belangrijkste 1-C-componenten (folaat, homocysteine, vitamine B₆ en B₁₂), bestudeerd, met daarbij de potentiële effecten van antidepressieve medicatie en de depressieve staat om meer inzicht te krijgen in de mogelijke associatie tussen het MTHFR-polymorfisme en MDD-R. Wij vergeleken het MTHFR C677T-polymorfisme samen met de hoofd 1-C-componenten bij patiënten met klinisch vastgestelde MDD-R (n=137) met leeftijd en gender gematchte gezonde controles (n=37). Ondanks onze specifieke patiëntenpopulatie met recidiverende depressie, konden we geen duidelijke
associatie vinden met de 1-C-cycle, met uitzondering van een hoger homocysteine en lager vitamin B₆ gehalte gedurende een depressieve episode. Dit suggereert dat 1-C-cycle wijzigingen in MDD-R afhankelijk zijn van de depressieve staat, waarschijnlijk veroorzaakt door een hoger niveau van acute (psychologische) stress. Het verlagen van stress zou een doel van de behandeling kunnen zijn om cardiovasculair risico te verminderen in deze populatie.

In hoofdstuk 5 (onderzoeksvraag 7) hebben we laten zien dat de relatie tussen de C677T MTHFR variant en de terugkeer van depressie gedurende een follow up van 5.5-jarig, gewijzigd werd door eerdere blootstelling aan traumatische ervaringen gedurende de kindertijd (TCEs) in een klinisch sample van 124 patiënten met MDD-R. Deze constatering werd gesteund door het feit dat bij een onafhankelijke replicatie sample van 665 gezonde individuen uit de algemene bevolking, de relatie tussen de C677T MTHFR variant en zelf gerapporteerde depressieve symptomen gewijzigd waren door TCEs. Samenvattend: T-allel dragers die blootgesteld zijn aan kindertrauma, zouden een hoger risico hebben op depressieve symptomen of recidivering van MDD.

Medische niet verklaarde fysieke symptomen
In hoofdstuk 6 (onderzoeksvraag 8) tonen we aan dat niet verklaarde medische fysieke symptomen (MUPS) een risico factor vormen voor terugval van recidiverende depressie gedurende 3.5 jaren. De relatie tussen MUPS en terugkeer van depressie kon niet verklaard worden door een associatie tussen aanhoudend hoge MUPS en omega (ω)-3 en −6 vetzuur (FA)-status en/of door het functionele polymorfisme in de promotor regio van het serotonine transporter gen (5-HTTLPR). Wij concluderen dat herkenning en vermindering van MUPS binnen dit subsample van patiënten in een vroeg stadium een (depressieve) terugval kan voorkomen.
Appendices

List of publications
Publications


**Papers in revision**


**Submitted papers**


Dutch papers
Appendices

Curriculum vitae
Anja Lok was born on the 9th of July 1970 in Bodegraven. From the age of 13 until she was 19 years old she was a professional jazz dancer. After finishing secondary school in Woerden, she began with the study Psychology at the University of Amsterdam in 1993. In 2000 she obtained her Master’s degree after completing the program of psychonomics and psychobiology of cognition and emotion. Directly after graduation she started her employment as a psychologist in the Onze Lieve Vrouwe Gasthuis (OLVG) hospital in Amsterdam. Together with Lucy Dijkman she developed a department specialized in the diagnostics and treatment of work related psychotrauma for health care organizations. Their OLVG-model for trauma care received the AWOZ-award from the Dutch Government and is implemented in other hospitals and ambulance organizations throughout the Netherlands and even internationally, in the South African TraumaClinic Capetown. In 2012, Anja Lok initiated that the boards of the OLVG and Arq Psychotrauma Expert Group combine their forces in trauma-work and are originating new intervention programs together.

In 1997 Anja Lok also started Medical School at the Academic Medical Center (AMC). In 2009 she finished her clinical rotations abroad under supervision of dr. Sander Kooij at the Department of Psychiatry of the Homerton University Hospital, a Foundation Trust based in the east London Borough of Hackney in the United Kingdom. In 2002 Anja Lok started her PhD project on biological factors in recurrent depression under supervision of Prof. dr. Aart Schene and Prof. dr. Claudi Bockting. In 2009 she began her psychiatry residency program at the AMC under supervision of Prof. dr. Aart Schene and dr. Jitschak Storosum and is expected to complete her training as a psychiatrist in 2015.
Appendices

Dankwoord
Dankwoord / Acknowledgements

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Time after Time; biological factors in the course of recurrent depression


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Time after Time; biological factors in the course of recurrent depression
Uitnodiging voor de verdediging van het proefschrift

**Time after Time; biological factors in the course of recurrent depression**

Anja Lok

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Singel 411 Amsterdam

Receptie na afloop van de plechtigheid

Paranimfen: Carin Meijer
Promotie: Anjalok@gmail.com
06 - 309 278 03
Conny van Niekerk
06 - 557 832 75