Biological Determinants of Depression: An epidemiological approach

NESE DIREK
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An epidemiological approach

Nese Direk
The work described in this thesis was performed within the framework of the Rotterdam Study at the Department of Epidemiology of the Erasmus Medical Center, Rotterdam, the Netherlands. The contribution of the study participants, the staff from the Rotterdam Study and all general practitioners and pharmacists is gratefully acknowledged.

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Biological Determinants of Depression:

*An epidemiological approach*

Biologische kenmerken bij depressive:
een epidemiologishe benadering

Na Rotterdam;
Desiderius Erasmus’ reisdoelen in Europa

After Rotterdam;
Desiderius Erasmus’ destinations in Europe

**Proefschrift**

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

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Chapter 1

Introduction
INTRODUCTION

Depression, in a broad sense, is a state of feeling sad. Nowadays, the term is applied to several clinically important conditions such as major depressive disorder, dysthymia, minor depression, sub-threshold depression and depressive symptoms that are not limited to a mood state of sadness. Major depressive disorder is a syndrome including several symptoms such as depressive mood, loss of interest, concentration problems and indecisiveness, recurrent suicidal ideation, sleep and appetite problems, feelings of guilt and worthlessness, lack of energy or irritability.

Major depressive disorder is one of the most common psychiatric disorders with a lifetime prevalence of ranging between 15-30% and estimated 12-month prevalence of around 5% . Also, this common disorder is a leading cause of the disease burden worldwide . Any insight into the etiology of depression would be beneficial to prevent the occurrence of disorder.

Since it was described in Ancient Greece, biological and non-biological factors underlying depression have been of interest. Several biological mechanisms including hormonal, vascular, immune and genetic mechanisms have been identified as contributing to the etiology of depression.

The hypothalamic-pituitary-adrenal axis and depression

One of the earliest findings of biological studies of depression is the disturbed regulation of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is the main neurohormonal system that regulates acute and chronic stress response. Physiological or psychological stress activates the hypothalamus resulting in secretion of the corticotrophin-releasing hormone (CRH) and therefore the adrenocorticotropic hormone (ACTH) in the anterior pituitary gland. In response to the ACTH secretion, cortisol is released from the adrenal glands. The axis is controlled with a negative feedback mechanism in which cortisol suppresses the release of the CRH and ACTH to prevent excess cortisol secretion when stressor has subsided. Cortisol secretion follows a diurnal rhythm. Before awakening, cortisol secretion starts increasing. It has a peak secretion 30-45 minutes after awakening and then it decreases during the day.

In early clinical studies, hypercortisolemia and a non-suppression of the HPA axis were found very frequently. Hence, several attempts has been made to use these findings as diagnostic tests in depression . However, further studies failed to replicate the initially very encouraging findings. Some found no difference in the HPA-axis activity in persons with depression and controls, while others found hypocortisolemia and an increased negative
feedback suppression of the HPA axis in depressed patients. Inconsistencies among the studies most likely stemmed from the different populations evaluated, cortisol collection methods, time points, and confounders that were not taken into account. Cortisol can be evaluated in different body specimen including blood, urine, and cerebrospinal fluid, all with different reference levels and variations on free cortisol levels.

More recently cortisol assessments in saliva and hair were developed. Measuring cortisol in saliva made it possible to use this method in large samples, as it is easy to apply in population-based studies. Also, saliva collection is easier than relying on plasma samples when assessing diurnal variation of cortisol, which requires several samples during the day. This helps researchers to overcome the main disadvantage of the one-time-point cortisol assessment in blood: the lack of accounting for diurnal variation of cortisol in large samples.

Another novel development that is relevant for cortisol research in population-based studies is the introduction of low doses of dexamethasone. Low doses of dexamethasone to test the negative feedback regulation of the HPA axis is unique, as interventional procedures are generally not used in such studies.

An epidemiological approach to cortisol research allows testing many confounders, interactions, and hypothesis-free genome-wide associations, which are important to understand how chronic diseases are related to disturbed cortisol regulation.

In this thesis, we aimed to assess diurnal variation, negative feedback control, and the genetics of the HPA axis in general population to explore some missing pieces in the puzzle of the association between the HPA axis functions and depression.

**Vascular factors and depression**

Recognizing the link between vascular factors and depression has a long history. Since the 19th century by the work of Durand-Fardel, a relationship between vascular disease such as atherosclerosis or stroke and depression was reported. More systematic studies were published by Alexopoulos in 1997. He introduced the ‘Vascular Depression’ hypothesis in which he proposed that ‘cerebrovascular disease precipitate, predispose and perpetuate’ late life depression. In the same year, Krishnan proposed the same concept focusing on vascular lesions such as white matter lesions (WMLs) using magneting resonance imaging (MRI) methods. Since then, a wide range of vascular depression studies has been performed. Until recently, a large body of studies has focused on the link between overt cerebrovascular diseases such as stroke, clinical infarcts and depression indicating a clear association bi-directionally.
Arguably, it is not sufficient to test the associations between vascular diseases, which come with severe disabling symptoms, and depression to prove vascular etiology of depression. Not unlikely, such disabling diseases can cause depression as a psychological reaction to disability rather than a vascular pathophysiological mechanism. For that reason, asymptomatic cerebrovascular disturbances, so called cerebral small vessel disease has been defined and tested in etiology studies of depression in the last decades. WMLs and cerebral infarcts and lacunes have been explored supporting vascular etiology in depression 11, 12.

To further our knowledge, relatively novel markers of cerebrovascular damage or functional component of the cerebrovascular system must be explored. A recent marker of cerebrovascular damage, cerebral microbleeds has not been tested in relation to depression. Similarly, the cerebral hemodynamics of depression have been studied only cross-sectionally in a few studies that show an impairment in blood flow 13-16.

In this thesis, we aimed to test the association of non-clinical cerebral small vessel disease with depression longitudinally in general population. Also, we aimed to see if amyloid pathology, which may affect the cerebrovascular functions, is related to depression.

**Genetic factors and depression**

Major depressive disorder is a heritable syndrome. Family studies have shown familial aggregation in depression. According to these studies, the heritability of MDD is between 28% and 44% 17, whereas the heritability estimate of the depressive symptoms is approximately 25% 18, 19. Depression is a genetically complex disorder in which common genetic variations with relatively small effect sizes determine the etiology. However, detecting such variants require very large sample size. The advent of human genome projects and genome-wide association studies (GWAS) in the 2000s, helped researchers to test multiple variants in genetically complex psychiatric traits. While GWAS of psychiatric traits such as psychosis, bipolar disorder, attention deficiency and hyperactivity disorder, autism yielded some loci, studies on depressive disorders were not successful until very recently (PGC MDD manuscript by Wray N.R. et al. submitted to Nature). One of the most important reasons was the relatively heterogeneous phenotype profile of depression and small sample sizes required to detect risk alleles. Two large consortia performed GWAS on MDD and depressive symptoms but no genome-wide significant loci were detected in these studies. In the current thesis, we aimed to widen the phenotype definition in depression and meta-analyzed these GWAS of MDD and depressive symptoms to increase the number of subjects and reach acceptable statistical power.

In this thesis, we revisit well-researched potential biological causes of depression with new technical and epidemiological methods. In Chapter 2 we explored cortisol in relation to
smoking and genetics to gain more insight on its biology. Also, we adapted an established concept, the dexamethasone suppression test, with an adapted method to test sociodemographic, health-related and psychiatric determinants in general populations. In Chapter 3 vascular depression hypothesis was revisited with advanced epidemiological designs and new imaging markers such as microbleeds and functional measures of cerebrovascular systems such as vasomotor reactivity. In Chapter 4 we present three GWAS of depression with different study designs and phenotype definitions.
REFERENCES


Chapter 2
Epidemiological studies of the HPA axis
Chapter 2.1

Short and long-term effects of smoking on cortisol in older adults

Nese Direk, Rachel S. Newson, Albert Hofman, Clemens Kirschbaum, Henning Tiemeier

ABSTRACT

We investigated concurrent as well as long-term effects of smoking on cortisol. The population consisted of 2,508 elderly adults. Current smokers, as opposed to former smokers, had higher basal cortisol levels and higher morning increases of cortisol. Overall, pack-years was related to morning cortisol rise, but this was accounted for by current smokers. Time since quitting was positively associated with a greater decline in daytime cortisol indicating that the effects of smoking remit. This suggests that smoking has short-term, rather than long-term, consequences on cortisol secretion patterns.
INTRODUCTION

Smoking is a commonly known risk factor for mental and physical health. However, the biological mechanisms for this are not always clear. Studies suggest that the effects on biological systems such as the endocrine system, may play a role for many chronic disorders. Cortisol is a stress hormone within the HPA axis, which is influenced by smoking and related to chronic diseases. Thus evaluating the association of smoking to cortisol may help to elucidate the mechanism by which smoking influences disease.

Cortisol follows a diurnal pattern, first rising 30-minutes after awakening and then declining throughout the day. Smoking acutely increases cortisol levels and it has been shown that this stimulation is dose-dependent. Studies in large samples of younger adults demonstrated that, relative to non-smokers current smokers have higher overall basal cortisol levels, greater morning increases, and lesser declines in cortisol over the day. Comparable results were reported in a middle age cohort study, in which current smokers had higher salivary cortisol levels and morning increases, when compared with former and never smokers, while there were no differences between former and never smokers.

Many smoking-related health problems occur after a long history of smoking, but subsequently decrease after smoking cessation (Office of the Surgeon General Report, 2004). As cortisol may mediate chronic health consequences of smoking, it is important to explore the effects of long-term smoking on cortisol. Older adults generally have longer smoking history than younger adults. Additionally, the duration since quitting is longer for former smokers in older adults. Although there is evidence for a short-term effect of smoking on cortisol in older adults, the long-term effects and the diurnal pattern have not been fully explored by examining long-term indicators of smoking. Therefore, the current study examined the association between short and long-term indicators of smoking and cortisol levels in older adults.

MATERIALS AND METHODS

The current study was based within the Rotterdam study, a population-based cohort of adults aged over 55 years which focuses on the occurrences and determinants of common chronic diseases. The data from the fourth follow-up round (2002-2004), in which salivary cortisol was sampled, was used in this cross-sectional study. The population consisted of 2,508 participants (mean age 74.9 ± 5.8 years, 58% female) whose smoking status data was complete, and who had at least one saliva sample. There were 297 current smokers (11.8%), 1,389 former smokers (55.4%), and 822 never smokers (32.8%).
Participants were asked about their smoking status, and current smokers were asked how many cigarettes they smoked daily and how long they had been smoking. Former smokers were asked about their smoking history with regard to the number of cigarettes they had smoked per day and the time since they had quit. Pack-years, a combination of duration and intensity of smoking, was subsequently calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person had smoked. The number of cigarettes smoked daily provides an indication of whether there is a dose-response relationship. Time since quitting makes it possible to determine whether the effect of smoking on cortisol was permanent, and pack-years is an indicator of long-term exposure. The latter is particularly pertinent to the current study, where, given the older age of the population, a longer life history could be obtained.

Because the cortisol level increases 30 minutes after awakening and decline throughout the day, multiple measures of cortisol levels during the day provide more valid information about daily cortisol release. Saliva samples were therefore collected on awakening (C1), after 30 minutes (C2), at 5pm (C3) and at bedtime (C4). HPA axis activity was evaluated by three summary measures. Total cortisol exposure throughout the day was tested by Area Under the Curve (AUC) given by the individual cortisol measurement on the y-axis and the time between the cortisol measurements on the x-axis. Cortisol Awakening Response (CAR) was used to investigate the morning increase 30 minutes after awakening. CAR was calculated as the difference between C2 and C1 over two. Diurnal decline was assessed by a slope, which was calculated by fitting a linear regression line for each participant, which predicted the cortisol values from time since awakening. A greater decline in daytime cortisol means that the slope of the regression line is steeper (higher or positive), whereas a lesser decline means that the slope is flatter (lower or negative).

Sex, age, and marital status were evaluated as covariates. The time between subsequent cortisol measurements within the same day was used as a covariate in the analysis of C2 and C3. Cognitive status was evaluated with the Mini Mental State Examination and used as a continuous variable. Education was scaled from primary education (1) to university level (7) and used as a continuous variable. Pharmacy records were used to collect data on systemic corticosteroid use.

The association between smoking and both the single and summary cortisol measures was evaluated. An ANOVA was conducted to determine whether smoking status (current, former, never) was associated with cortisol. An ANCOVA was performed to test the association between smoking status and cortisol, adjusting for sex, age, marital status, cognitive status, education, corticosteroid use, and time between cortisol measures (for C2 and C3). The pair-wise comparisons between the three smoking groups were evalu-
ated using a Bonferroni correction with an adjusted $p$ value of .0167 (three tests were conducted; .05/3) for statistical significance. A series of adjusted linear regressions were conducted to examine the association between the number of daily cigarettes, pack-years, time since quitting and cortisol. All analyses were adjusted for sex, age, marital status, cognitive status, education, corticosteroid use, and time between cortisol measures. To enhance comparability across the results, missing values of covariates were imputed by using the Expectation-Maximization Algorithm. The maximum percent of missing values was 12% (corticosteroid use). Missing variables were imputed on the basis of the entire baseline population (n=3,539).

RESULTS

Participants’ characteristics according to smoking status are presented in Table 1. Current smokers reported an average of $12.4 \pm 7.4$ daily cigarettes. Mean pack-years was $33.6 \pm 21.4$ in current smokers and $25.4 \pm 23.5$ in former smokers ($p < .001$). Mean time since quitting for former smokers was $28.2 \pm 13.6$ years.

Table 1. Participant characteristics by smoking status.

<table>
<thead>
<tr>
<th></th>
<th>Current smoker</th>
<th>Former smoker</th>
<th>Never smoker</th>
<th>$p^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± (SD)</td>
<td>73.4 ± (5.3)</td>
<td>74.9 ± (5.6)</td>
<td>75.4 ± (6.2)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Gender (female), n (%)</td>
<td>170 (57.2)</td>
<td>596 (42.9)</td>
<td>693 (84.3)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Marital status (married), n (%)</td>
<td>161 (54.2)</td>
<td>941 (67.7)</td>
<td>454 (55.2)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Education (primary school only), n (%)</td>
<td>48 (16.2)</td>
<td>159 (11.4)</td>
<td>135 (16.4)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Mini Mental State Examination, mean ± (SD)</td>
<td>27.5 ± (1.9)</td>
<td>27.6 ± (2.3)</td>
<td>27.4 ± (2.3)</td>
<td>.20</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²), mean ± (SD)</td>
<td>26.1 ± (3.7)</td>
<td>27.6 ± (3.9)</td>
<td>27.6 ± (4.2)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Number of cigarettes smoked daily, mean ± (SD)</td>
<td>12.4 ± (7.4)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Pack-years, mean ± (SD)</td>
<td>33.6 ± (21.4)</td>
<td>25.4 ± (23.5)</td>
<td>N/A</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Time since quitting (years), mean ±(SD)</td>
<td>N/A</td>
<td>28.2 ± (3.6)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

$^a$ $p$ values are overall values.

Single cortisol levels and summary measures by smoking status were compared by ANCOVA. Results of descriptive and inferential analyses of cortisol among groups are presented in Table 2. Adjusted analyses demonstrated that smoking status significantly affects $C_2$, $C_3$, $C_4$, AUC, and CAR. Post-hoc comparisons using a Bonferroni correction indicated that current smokers had higher mean $C_2$, $C_3$, $C_4$, AUC, and CAR ($p < .01$) than former and never smokers. There were no significant differences in any cortisol measures between never and former smokers.
Table 2. Descriptive and inferential analyses of saliva cortisol levels and three summary measures by smoking status

<table>
<thead>
<tr>
<th></th>
<th>Descriptive analyses</th>
<th>Inferential analyses</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>unadjusted mean ± (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Unadjusted</td>
<td></td>
<td></td>
<td>Adjusted</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Current smoker n= 297</td>
<td>Former smoker n= 1,389</td>
<td>Never smoker n= 822</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Salivary cortisol (nmol/liter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at awakening (C1)</td>
<td>14.5 ± (9.6)</td>
<td>14.9 ± (8.6)</td>
<td>14.2 ± (8.3)</td>
<td>2, 2297</td>
<td>1.7</td>
<td>.20</td>
<td>2, 2295</td>
<td>.3</td>
<td>.78</td>
<td></td>
</tr>
<tr>
<td>awakening +30 min (C2)</td>
<td>21.2 ± (11.6)</td>
<td>18.2 ± (10.2)</td>
<td>17.6 ± (9.8)</td>
<td>2, 2382</td>
<td>13.1 &lt;.001</td>
<td>2, 2185</td>
<td>10.7 &lt;.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 pm (C3)</td>
<td>5.1 ± (3.8)</td>
<td>4.3 ± (3.6)</td>
<td>4.2 ± (3.8)</td>
<td>2, 2417</td>
<td>7.2</td>
<td>.001</td>
<td>2, 2181</td>
<td>7.4</td>
<td>.001</td>
<td></td>
</tr>
<tr>
<td>bedtime (C4)</td>
<td>2.7 ± (2.4)</td>
<td>2.3 ± (2.6)</td>
<td>2.4 ± (2.7)</td>
<td>2, 2307</td>
<td>2.5</td>
<td>.08</td>
<td>2, 2096</td>
<td>5.0</td>
<td>.009</td>
<td></td>
</tr>
<tr>
<td>Cortisol Awakening Response (nmol/liter)</td>
<td>3.4 ± (6.0)</td>
<td>1.6 ± (5.4)</td>
<td>1.6 ± (5.0)</td>
<td>2, 2192</td>
<td>12.6 &lt;.001</td>
<td>2, 2016</td>
<td>13.0 &lt;.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area Under the Curve (nmol/liter)</td>
<td>9.5 ± (4.2)</td>
<td>8.2 ± (3.8)</td>
<td>8.1 ± (3.7)</td>
<td>2, 2022</td>
<td>12.5 &lt;.001</td>
<td>2, 2186</td>
<td>12.2 &lt;.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope (nmol/liter/h)</td>
<td>-0.78 ± (0.64)</td>
<td>-0.82 ± (0.56)</td>
<td>-0.80 ± (0.55)</td>
<td>2, 2063</td>
<td>.6</td>
<td>.60</td>
<td>2, 2057</td>
<td>.6</td>
<td>.56</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Association between short and long-term indicators of smoking and summary cortisol measures

<table>
<thead>
<tr>
<th>Outcome cortisol measure</th>
<th>Former and current smokers</th>
<th>Current smokers</th>
<th>Former smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pack-years</td>
<td>Daily amount of cigarette</td>
<td>Pack-years</td>
</tr>
<tr>
<td></td>
<td>β (95% CI)</td>
<td>p</td>
<td>β (95% CI)</td>
</tr>
<tr>
<td>Cortisol Awakening Response (nmol/liter)</td>
<td>.020 (.007; .033)</td>
<td>.003 .076 (-.031; .183)</td>
<td>.20 .038 (-.001; .077)</td>
</tr>
<tr>
<td>Area Under the Curve (nmol/liter)</td>
<td>.005 (-.005; .014)</td>
<td>.35 .037 (-.047; .120)</td>
<td>.40 .018 (-.012; .049)</td>
</tr>
<tr>
<td>Slope (nmol/liter/hour)</td>
<td>.001 (.000; .003)</td>
<td>.06 .003 (-.009; .015)</td>
<td>.60 .001 (-.003; .006)</td>
</tr>
</tbody>
</table>

Note: A linear regression was performed and adjusted for age, sex, time intervals, marital status, education, mini mental state examination scores, and corticosteroid use. β denotes the change of cortisol measure per smoking category, e.g., for per pack-years, the Cortisol Awakening Response increases .02 nmol/liter in former and current smokers.
A series of adjusted linear regressions were conducted to examine the association between cortisol and: the number of daily cigarettes, pack-years, and time since quitting. Amongst those with a history of smoking, pack-years was associated with higher CAR ($\beta = .020$; 95% confidence interval (CI)= .007-.033; $p< .01$). Interestingly, when former and current smokers were evaluated separately, this association decreased for former smokers ($\beta = .012$; 95 % CI= -.002 , .026; $p = .08$) and increased for current smokers ($\beta = .038$; 95 % CI= -.001, .077; $p = .06$). However, the associations were not statistically significant. Finally, a longer duration since quitting was associated with a greater decline in daytime cortisol ($\beta = -.003$; 95 % CI= -.005, .000; $p = .04$) (Table 3).

**DISCUSSION**

In this cross-sectional study, smoking was associated with increased cortisol levels throughout the day and a greater morning increase. This association was independent of demographic and health-related factors. Cortisol levels have previously been found to be high in current younger adult smokers 4. Previously in a middle aged occupational cohort study cortisol levels and a morning increase had been compared in current, former, and never smokers. In that study, cortisol release and CAR had been found to be higher in current smokers in comparison with former and never smokers 6. Thus, the current study supports the findings of prior research and extends it to an older age group. Moreover, the results indicate that smoking increases CAR. However, there are conflicting results 11, 12. A morning increase of cortisol has been considered as a distinct phenomenon than diurnal profile of cortisol. It is most likely associated with the process of awakening (e.g. orientation, activation of memory in the morning) 13. Because the population used in this study consisted of older adults, poor cognitive function may confound the association between smoking and CAR. However, this is unlikely, because adjustment for Mini Mental State Examination scores did not change the results.

Previous studies discussed whether the effect of smoking on cortisol changes after quitting; however, this had not been directly tested 6. Results of the current study suggest that the effects of smoking on cortisol are not permanent. First, current smokers in this study had significantly higher overall cortisol levels and greater CAR than former and never smokers, whereas former and never smokers did not differ. This suggests that the effect of smoking on cortisol recedes after quitting. Second, smoking history as measured in pack-years was positively related to a higher CAR; however, this effect was brought about by current smoking status rather than by smoking history. Third, time since quitting was associated with a greater decline in daytime cortisol which strongly suggests a remitting effect of smoking on cortisol. Previous studies compared the effect of short and mid-term smoking
cessation on cortisol levels. The present study demonstrates that there is a dose-response effect of smoking cessation depending on the time since quitting. In other words, a decline in cortisol over the day normalizes over a longer period of time.

Disturbance of cortisol release is an important etiological factor for many chronic situations, including atherosclerosis, high blood pressure and low-grade inflammation. Changes in cortisol release can be a causal factor for various smoking-related chronic diseases. However, the risk of smoking-related diseases decreases after quitting. This observation suggests that some of the potential pathological mechanisms that play a role in smoking-related diseases may not continue after quitting. The non-permanent effect of smoking on cortisol in this study supports this observation. However, it must be kept in mind that many cortisol-related changes may be irreversible once pathology occurs, for example atherosclerosis, which generally cannot be reversed.

The mechanism underlying the link between smoking and HPA axis remains unknown. However, it has been suggested that nicotine acts on the nicotinic acetylcholine receptors (nAChRs) in the hypothalamus and affects HPA axis functions. As a distinct phenomenon, CAR has been associated with the hippocampal networks, and therefore memory and orientation after awakening. Nicotine may play role in the hippocampus by acting on nAChRs and thus, may alter CAR in older adults. However, to date no study has explored this mechanism.

The sampling adherence was not evaluated in the current study due to lack of reliable awakening time data, which must be considered when interpreting these results. This may lead to underestimation of the effects on cortisol levels. This is a common problem, occurring in the majority of the population-based studies of cortisol. However, if it is not strongly associated with smoking, poor sampling adherence does not easily explain the observed effects.

In summary, this study, its strengths including a large sample size, a population-based background, multiple daily cortisol measurements, longer smoking history, and long-term indicators of smoking, suggests that smoking affects daytime cortisol levels and a morning rise only in current smokers. Further research should explore to what extent quitting diminishes chronic consequences of smoking caused by elevated HPA axis activity in smokers.
REFERENCES


Chapter 2.2

The very low-dose dexamethasone suppression test in the general population: A cross-sectional study

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ABSTRACT

Determinants of the HPA axis functioning are increasingly explored in the population-based studies. However, functional tests measuring the negative feedback of the HPA axis cannot easily be implemented into large observational studies. Furthermore, high doses of dexamethasone often completely suppress the HPA axis in healthy persons. This study aimed to detect the effects of the health, lifestyle and sociodemographic factors, psychiatric problems and cognitive functions on the negative feedback of the HPA axis using a very low-dose (0.25 mg) dexamethasone suppression test (DST). We evaluated the associations of several determinants with the saliva cortisol concentrations after dexamethasone intake in a confounder-adjusted model also corrected for baseline saliva cortisol concentrations in the Rotterdam Study, a large population-based study (N=1822). We found that female sex, low income, lack of exercise, instrumental disability and smoking were all independently associated with stronger suppression of the HPA axis. Even though there were no linear associations between psychiatric measures and cortisol suppression, we found that depressive symptoms and anxiety disorders were more common in persons with non-suppression of cortisol. Conversely, psychotropic medication use was related to enhanced suppression of cortisol after DST. In this large study, we found that female gender, low socioeconomic status and poor health were all related to suppression of the HPA axis. Non-linear associations were detected between the suppression of the HPA axis and common psychiatric disorders in community-dwelling persons.
INTRODUCTION

The HPA axis controls the stress response in the body. To explain the relation between diurnal HPA axis functioning and metabolic or stress-related disorders in large cohorts of healthy persons, researchers relied on single morning blood measures or used repeated saliva sampling. Different measures of the HPA axis such as cortisol awakening response, diurnal decline and total cortisol exposure over the day have been assessed using mostly saliva. More recently, cortisol assessment in hair became possible. In contrast, negative feedback of the HPA axis is rarely tested in non-clinical studies.

In clinical populations, the negative feedback of the HPA axis is assessed by a DST test requiring oral administration of a dose of dexamethasone. Cortisol secretion after dexamethasone intake is typically suppressed. A non-suppression indicates hypercortisolemia but DST proved very difficult to implement in population-based studies.

The DST was originally designed to diagnose patients with Cushing’s Syndrome using 1 mg or higher doses of dexamethasone with a cut-off value for the plasma cortisol to distinguish between non-suppressors and suppressors. A dose of 1 mg in the conventional DST fully suppresses the HPA axis in most persons from a non-clinical population. Furthermore, the traditional “black and white” outcome definition of the negative feedback of the HPA axis masks any variation across the continuum of HPA axis reactivity. To this aim, cut-points were defined for the conventional DST, which relies on plasma cortisol to define suppression. In this population-based study, we tested the determinants of the negative feedback of the HPA axis with a very low-dose DST (0.25 mg) and assessed the outcome (suppression) continuously.

Different determinants of the suppression or non-suppression after DST have been explored in the last decades. Over the years, the DST became perhaps the most common function test in clinically depressed persons. However, like in other psychiatric disorders including post-traumatic stress disorders, anxiety disorders, eating disorders, psychosis, personality traits, results of this test are inconsistent.

The majority of research has been performed in clinical samples with small sample sizes. Clinical studies are generally more efficient to test associations of an exposure and disease than population-based cohort studies. However, evaluating background variables is generally not possible in clinical studies. Often, only few background variables are assessed and background variables are related to disease (case) status, which gives rise to selection bias. Sometimes if matching is performed in clinical studies to control for the effect of a potential confounder. It is not possible to explore the importance of this matching variable.
for cortisol suppression anymore. Yet, evaluating determinants and potential confounders of the negative feedback of the HPA axis is important as numerous psychological and physiological stressors can potentially affect this system. Also, knowledge about the possible confounders of the cortisol suppression is mostly derived from the measurement of single cortisol levels or diurnal cortisol secretion. Thus, large population-based studies are needed to explore the determinants of the reaction to the DST. Until now, only one cohort explored the determinants (non-psychiatric) of negative feedback of the HPA axis using the low-dose DST (0.5 mg dexamethasone)\(^5\). In this study (n=455), a non-suppression of cortisol in the low-dose DST was observed in smokers, less active persons and sampling on a weekday. However, 86.4\% of the study population was below the study-specific cut-off for cortisol suppression suggesting that 0.5 mg might be a too high dose to evaluate functioning of the HPA axis in the general population.

Here, we aimed to explore the most important determinants of the negative feedback sensitivity of the HPA axis using a very low-dose (0.25 mg dexamethasone) DST in a large population-based study evaluating several determinants of health, lifestyle and sociodemographic factors, psychiatric problems and cognitive functions.

MATERIALS AND METHODS

The Rotterdam Study is a prospective population-based cohort investigating occurrence and determinants of chronic diseases. It has been approved by the institutional review board of the Erasmus University Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports. All participants provided written informed consent after complete description of the Rotterdam Study.

The current study was embedded in the third cohort of the Rotterdam Study (RS-III), which was initiated in 2006 and ended in 2008. Details regarding the study design of the RS-III are provided elsewhere\(^20\). This cohort included 3,932 subjects aged 45 to 54 years and those who had moved to the study district regardless of age. Among those, 3,247 (82.6 \%) participated in the day-curve saliva cortisol collection. We invited these participants for the DST and 2076 (63.9 \%) agreed to also take a dexamethasone tablet and to collect two additional saliva samples. Fifty-eight participants did not report time of sampling of a saliva sample, and 130 did not produce enough saliva to analyze cortisol. Further, we excluded participants from all analyses if the time difference between the two samples deviated more than 3 h from the specified 24h (n=59) (see sensitivity analysis for more stringent exclusion criteria based on sampling interval). Finally, we excluded participants, who reported the use of systemic corticosteroids (n=7) leaving 1822 participants for the
current study. When compared to the participants (n=1822), non-participants who did not participated in the DST or were excluded from the analyses (n=1425) were more likely to be male (p=.007) and younger (p < .001).

Assessment of the Dexamethasone Suppression Test

Participants were asked to collect saliva samples at home using the Salivette sampling devices (Sarstedt, Nümbrecht, Germany). They received detailed oral and written instructions with particular emphasis on the importance of compliance to sampling time. Participants were instructed to collect the first saliva sample at 8 am, to take the dexamethasone pill at 11 pm at the same day, and to collect the second saliva sample the next morning at 8 am. This approach was chosen as similar sampling times have commonly been used in clinical and population-based studies. High early morning levels of cortisol maximize the power to detect differences. Additionally participants were instructed not to eat and not to brush their teeth 15 minutes before collecting the samples and to report the exact time and date of the sampling and dexamethasone intake on a form provided by the researchers. More detail about the instructions given to the participants are provided in the supplement.

We kept the Salivettes at -80 °C until they were sent to the Laboratory of Biopsychology, Technical University of Dresden, Germany. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Hamburg, Germany). Intra-assay and interassay coefficients of variation were less than 6% and 9%, respectively (obtained for control samples with average cortisol levels 4.1 nmol/l and 29.5 nmol/l). The lower limit of detection was 0.4 nmol/liter. Saliva cortisol levels were below the detection limit in one person for the second saliva sample in our sample. A level of zero nmol/l was used for this participant. Before dexamethasone intake, none of the participants had a cortisol level below the detection limits in the current study sample.

Assessment of Determinants

We selected possible determinants of cortisol suppression after DST on the basis of previous literature and grouped them into four categories similar to the previous epidemiological studies: Sociodemographic indicators, health and lifestyle variables, psychiatric problems and cognitive functions and sampling variables.

Sociodemographic indicators were age, gender, education and monthly household income. Educational attainment was assessed in seven categories from primary education to university level on the basis of the Standard Classification of Education. In this study, we grouped education into three categories including low, intermediate and high. Monthly net household income was reported in 13 ordinal categories (from < 500 € to ≥ 2900 €) and analyzed continuously.
We studied body mass index, smoking status, disability, exercise, diabetes mellitus and hypertension as indicators of health and lifestyle. Height (meters) and weight (kilograms) were measured and body mass index was calculated as kg/m$^2$. Smoking status was coded in three categories as never, former, and current smoker. Different dimensions of disability were evaluated using Activities of Daily Living (ADL) from the Stanford Health Assessment Questionnaire and Instrumental Activities of Daily Living (iADL) scales. We used a pre-defined cut-off ADL score of 0.5 to define a mild or severe disability. A cut-off score of 0.23, which is one standard deviation of the mean iADL score was used to define persons with instrumental disability. Exercise was evaluated using a single item from a questionnaire that was prepared by researchers evaluating sport habits on a regular basis. Diabetes mellitus was diagnosed if participants were on antidiabetic medication or if their fasting blood glucose concentrations were 200 mg/dL or higher. Systolic and diastolic blood pressures were measured twice in the resting state with a random-zero sphygmomanometer and the mean of two consecutive measurements was calculated. Hypertension was defined as a systolic blood pressure $\geq 140$ mm Hg or a diastolic blood pressure $\geq 90$ mm Hg or the use of antihypertensive medication.

We examined general cognitive functions, clinically relevant depressive symptoms, depressive disorders, anxiety disorders, psychotic experiences, and psychotropic medication as psychiatric variables. General cognitive functions were measured with the Mini–Mental State Examination (MMSE) and a g-factor. This g-factor was derived from principal component analysis of processing speed, executive function, verbal fluency, verbal recall and recognition, visuospatial ability and fine motor skills. Persons with an MMSE score of less than 24 were categorized as cognitively impaired. Depressive symptoms were evaluated with the Center for Epidemiological Studies-Depression (CES-D) scale and a cut-off of 16 was used to detect participants with clinically relevant depressive symptoms. All participants with clinically relevant depressive symptoms were invited for a semi-structured clinical interview, the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) to detect DSM-IV-TR diagnosis of depressive disorders. These interviews were performed by trained clinicians and psychologists in close proximity in time to screening (on average 2 weeks). Anxiety disorders were diagnosed with an adapted version of the Munich version of the Composite International Diagnostic Interview (M-CIDI). All DSM-IV based anxiety disorders (except obsessive-compulsive disorder and post-traumatic stress disorder) were assessed and studied as a categorical variable. Psychotic experiences were evaluated with the Community Assessment of Psychic Experiences (CAPE)-positive experiences scale (20 self-reported items). Each item assesses frequency and distress related to the psychotic experience. Data on psychotropic medication use was obtained from pharmacy records.
As sampling variables, we considered cortisol concentrations before dexamethasone intake and the day of the sampling. The day of the sampling was categorized as weekday or weekend. In addition, all analyses were adjusted for the times of saliva sampling before and after dexamethasone intake.

**Statistical Analyses**

We used the natural-log function to transform the cortisol concentrations after dexamethasone intake because of the skewed distribution (Figures A and B in S1 file). We present effect estimates only for the transformed values. First, we examined the relation of the individual determinants with cortisol concentrations after dexamethasone intake using linear regression analysis. The basic model was adjusted for age, sex and cortisol concentrations assessed the previous day. This adjustment is considered a more reliable method than change score (cortisol after - cortisol before) and percentage change ([cortisol after - cortisol before / cortisol before]*100) methods. Moreover, a simulation study showed that analysis using percentage change has very low statistical power when compared to the model in which a post-test value is used as an outcome and a pre-test value is used as a confounder.\(^{33,34}\). Second, we fitted a multivariable model in which all determinants used in the current study were included in the same model. Because of the well-known effect of body weight on the associations of gender and smoking status with cortisol, these analyses (both basic and multivariable models) were additionally adjusted for body weight.

In addition, non-linear associations of psychiatric traits were explored contrasting the highest and lowest tenth percentiles versus the middle group. The highest tenth percentile was used to define participants with non-suppression and the lowest tenth percentile was used to define participants with enhanced suppression.

All analyses were further adjusted for time of the first and second cortisol samplings.

In addition, we performed sensitivity analyses to further test the possible effect of the time interval between the first and second cortisol sampling. We reran the multivariable model including only participants who complied fully with a time interval of 24 ± 1 hours (n=1632) between the moments of saliva sampling and also including only those with a time interval of 24 ± 2 hours (n=1766).

In a supplementary analysis, we tested the associations of the selected variables with the baseline cortisol concentrations (i.e., the concentrations before dexamethasone intake) to explore the associations with the non-suppressed basal cortisol secretion. These contrasting analyses help to evaluate which findings of the very-low dose dexamethasone test are specific for cortisol suppression.
To facilitate the comparison of the effect estimates of the various variables independent of the units of variables, we reported the standardized regression coefficients. All determinants had less than 10% of missing values. To enhance the comparability across the results, we imputed missing values of the determinants using the Expectation-Maximization Algorithm.

SPSS version 20 (SPSS, Inc., Chicago, IL) was used for all analyses.

RESULTS

The participant characteristics are presented in Table 1. The mean age was 57.8 years (standard deviation [SD] = 6.8 years) and 59.6% (n=1086) of the study population was female. The mean cortisol concentration before dexamethasone intake was 15.6 nmol/L (SD=10.9) and the mean concentration after dexamethasone intake was 7 nmol/L (SD=8.5). Gender specific mean cortisol concentrations before and after dexamethasone intake are shown in Fig 1.

Table 2 shows the results of the associations between determinants and the negative feedback control of cortisol as indicated by cortisol concentrations after the very low-dose DST adjusted for baseline cortisol concentration. The baseline cortisol concentrations

<table>
<thead>
<tr>
<th>Table 1. Characteristics of the study population</th>
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<tbody>
<tr>
<td><strong>Characteristics</strong></td>
</tr>
<tr>
<td><strong>Sociodemographic indicators</strong></td>
</tr>
<tr>
<td>Age, years, mean (SD)</td>
</tr>
<tr>
<td>Women, n (%)</td>
</tr>
<tr>
<td>Low education, n (%)</td>
</tr>
<tr>
<td>Paid job, n (%)</td>
</tr>
<tr>
<td>Net income, median (range)</td>
</tr>
<tr>
<td>Married, n (%)</td>
</tr>
<tr>
<td><strong>Health &amp; lifestyle variables</strong></td>
</tr>
<tr>
<td>Mild/severe disability, n (%)</td>
</tr>
<tr>
<td>Instrumental disability, n (%)</td>
</tr>
<tr>
<td>Regular exercise, n (%)</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
</tr>
<tr>
<td>Current smoker</td>
</tr>
<tr>
<td>Former smoker</td>
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</tbody>
</table>
before dexamethasone intake were substantially and positively associated with cortisol concentrations after dexamethasone intake ($\beta = 0.41, \ p < .001$); i.e., high cortisol concentrations before dexamethasone intake were related to high cortisol concentrations after dexamethasone intake.
Cortisol suppression after dexamethasone intake was higher in women when compared to men ($\beta = -0.16, p<.001$). As women were lighter than men (74.38 kg, SD= 13.77 vs. 88.12 kg, SD= 13.4), we corrected the basic and mutually adjusted models for body weight. However, the gender difference in cortisol levels remained significant (results not shown). Older age was also associated with stronger cortisol suppression after dexamethasone intake ($\beta = -0.05, p=.017$), but this association was not significant in the multivariable model in which several health-related variables were included. Low income was related to stronger cortisol suppression after very low-dose DST in the multivariable model ($\beta = 0.06, p=.020$). Indicators of poor health such as instrumental disability ($\beta = -0.07, p=.002$), lack of exercise ($\beta = -0.05, p=.019$) and smoking ($\beta = -0.07, p=.002$) were all related to increased negative feedback control of cortisol in the multivariable models. To eliminate the possible effect of weight on the association of smoking status and cortisol suppression, we adjusted the basic and mutually adjusted models for weight instead of BMI; results remained significant (results not shown). Poor global cognition function ($\beta = 0.07, p=.01$) was related to stronger cortisol suppression after dexamethasone intake in the basic model. However, this association was not significant in the multivariable model. Use of psychotropic medications was related to enhanced cortisol suppression following...
Table 2. Relations of determinants with cortisol concentrations after dexamethasone intake

<table>
<thead>
<tr>
<th>Sociodemographic indicators</th>
<th>Cortisol concentration after dexamethasone intake</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Basic model&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mutually adjusted model&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>B</td>
</tr>
<tr>
<td>Age (years)</td>
<td>-0.05</td>
<td>-0.01</td>
</tr>
<tr>
<td>Sex (0=male, 1=female)</td>
<td>-0.16</td>
<td>-0.28</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>-0.01</td>
<td>-0.02</td>
</tr>
<tr>
<td>Intermediate</td>
<td>-0.05</td>
<td>-0.08</td>
</tr>
<tr>
<td>High (reference)</td>
<td>(reference)</td>
<td>(reference)</td>
</tr>
<tr>
<td>Net income (ranked 1 to 13)</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>Health &amp; lifestyle variables</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild/severe disability (0=no, 1=yes)</td>
<td>-0.03</td>
<td>-0.06</td>
</tr>
<tr>
<td>Instrumental disability (0=no, 1=yes)</td>
<td>-0.07</td>
<td>-0.18</td>
</tr>
<tr>
<td>Regular exercise (0=yes, 1=no)</td>
<td>-0.08</td>
<td>-0.13</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker (reference)</td>
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<td></td>
</tr>
<tr>
<td>Former smoker</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Current smoker</td>
<td>-0.09</td>
<td>-0.19</td>
</tr>
<tr>
<td>Body mass index (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>Diabetes mellitus (0=no, 1=yes)</td>
<td>-0.04</td>
<td>-0.11</td>
</tr>
<tr>
<td>Hypertension (0=no, 1=yes)</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Psychiatric problems &amp; cognitive functions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressive symptom score</td>
<td>-0.10</td>
<td>-0.001</td>
</tr>
<tr>
<td>Clinically relevant depressive symptoms (0=no, 1=yes)</td>
<td>51</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Cortisol concentration after dexamethasone intake</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td></td>
<td>Basic model</td>
<td>Mutually adjusted model</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>B</td>
</tr>
<tr>
<td>Major depressive disorder (0=no, 1=yes)</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td>Anxiety disorders (0=no, 1=yes)</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>MMSE score</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Cognitive impairment (0=no, 1=yes)</td>
<td>-0.01</td>
<td>-0.05</td>
</tr>
<tr>
<td>The g-factor of the cognitive tests</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Psychotic experiences (CAPE-positive items)</td>
<td>-0.04</td>
<td>-0.03</td>
</tr>
<tr>
<td>Psychotropic medications (0=no, 1=yes)</td>
<td>-0.04</td>
<td>-0.10</td>
</tr>
</tbody>
</table>

**Sampling variables**

<p>| | |</p>
<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Day of the sampling (0=weekend, 1=weekday)</td>
<td>-0.004</td>
</tr>
<tr>
<td>Cortisol before dexamethasone intake, nmol/L</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*Adjusted for cortisol concentrations before dexamethasone intake, age and sex

*Mutually adjusted model when appropriate.

**Abbreviations:** β, standardized beta; B, unstandardized beta; CI, Confidence Interval of the unstandardized beta; MMSE, Mini Mental State Examination; CAPE, The Community Assessment of Psychic Experience
dexamethasone intake in the age- and gender adjusted model ($\beta = 0.04, p= .05$). This association disappeared after adjustment in the multivariable model. Clinically relevant depressive symptoms, depressive disorders, anxiety disorders, psychotic experiences were not related to the negative feedback control of cortisol in any of the models. To test non-linear associations of psychiatric traits with cortisol suppression, we defined non-suppression and enhanced cortisol suppression patterns. Depressive symptom scores (OR = 1.02, $p = .03$), clinically relevant depressive symptoms (OR = 1.93, $p = .01$) and anxiety disorders (OR = 2.42, $p = 0.001$) were related to non-suppression. In contrast, psychotropic medication use was related to enhanced cortisol suppression (OR = 2.08, $p < .001$) (Table B in the supplement).

The multivariate model explained 22% of the variance ($F(18, 1803)= 29.46, p< .001$).

Adjustment for time of sampling did not change the results in any of the models (data not shown). Day of the sampling was not associated with cortisol suppression after dexamethasone intake.

To contrast the results with the determinants of basal cortisol secretion, we also examined the relation of these variables with cortisol concentration before dexamethasone intake. In this analysis, a different pattern of associations emerged: instrumental disability, BMI and cognition were all related to basal cortisol concentrations (Table A in the supplement). However, sex, smoking, low income and lack of exercise were not associated with basal cortisol levels. In summary, only instrumental disability determined both the degree of cortisol suppression and basal cortisol concentrations.

The sensitivity analyses excluding poor self-reported compliers suggests that excluding people with poor compliance to the DST protocol did not change the effect estimates meaningfully (Figure C in the supplement.).

**DISCUSSION**

In this large population-based study, we found that female sex, low income, lack of exercise, instrumental disability and smoking were all associated with stronger suppression of the HPA axis as determined by lower cortisol concentrations after a very low-dose DST.

We observed that women had stronger suppression of the HPA axis than men. Our result is consistent with a previous report in which an increased negative feedback control of cortisol was found in women without psychopathology. This gender difference might be related to circulating gonadal steroids in women. The mechanisms are not well understood;
it is known that high concentrations of estradiol may alter the negative feedback control of cortisol leading to high cortisol levels\textsuperscript{35}. In contrast, a decrease in estradiol levels during the menopause, as probably experienced by the women in our study (mean age of 57.8 years), may cause stronger suppression of the HPA axis\textsuperscript{36, 37}. The gender difference might also result from weight differences as, on average, women are lighter than men. This difference could explain a greater effect of dexamethasone in lean people (i.e. women in our study) leading to more cortisol suppression. However, our results did not change when we adjusted for body weight. Low income, an indicator of low SES, was also associated with stronger suppression of the HPA axis. It is known that ongoing financial strain may give rise to chronic stress\textsuperscript{38} which has repeatedly been linked to stronger suppression of the HPA axis. Low income has previously been associated with high\textsuperscript{39-41} and low cortisol concentrations in studies with single time-point cortisol sampling\textsuperscript{42}. However, our results cannot be compared straightforwardly with the findings of these studies, because income was not tested in relation to the negative feedback of the HPA axis in the general population.

Health and lifestyle variables including physical disability, lack of exercise, and smoking were associated with increased negative feedback sensitivity. Physical activity is considered as a direct stimulator of the HPA axis\textsuperscript{43}. In a previous population-based study, however, physical activity as measured by metabolic equivalent (MET) of the number of calories expended per minute in an activity and cortisol level after dexamethasone intake were not related. In the same study, less MET was related with lower cortisol suppression ratio indicating a non-suppression\textsuperscript{5}. In the current study, the questions on physical activity addressed whether a person performed at least one sport activity on a regular basis. Like in most measures, a positive answer indicates physical activity and fitness but may also reflect general health. Good health is a prerequisite for sports participation and this may contribute to the inconsistency between two studies.

Smoking has been associated with a hyperactive HPA axis in previous population-based studies\textsuperscript{5, 44, 45}, although habitual smokers have low basal cortisol concentrations. Habitual smokers develop desensitization to nicotine exposure at the acetylcholinergic receptor level. Unless the nicotine exceeds a certain threshold, the HPA axis may not be activated at the hypothalamic level, which may lead to a hyper-suppression of the HPA axis in habitual smokers in line with our observation\textsuperscript{46}. Additionally, it was shown that habitual smokers have an attenuated response to the stress, which might be due to the stronger suppression of the HPA axis\textsuperscript{46, 47}. As mentioned previously, body weight may affect the pharmacodynamics of dexamethasone. Smokers are generally leaner than non-smokers and difference in weight might affect the association between smoking status and cortisol suppression. However, our results remained essentially unchanged when the analysis was adjusted for weight.
None of the characteristics associated with the HPA axis suppression showed any association with morning cortisol levels alone, except instrumental disability. This suggests that the degree of cortisol suppression after the pharmacological stimulation cannot be inferred from basal cortisol levels.

Cognitive functions were related to the negative feedback of the HPA axis. Poor global cognitive functioning as assessed with the g-factor was related to low cortisol levels after dexamethasone intake at the age- and gender-adjusted model. However, this association was not significant in the multivariable model, in which analyses were adjusted for physical health and mental health items. Still, it is important to consider global cognitive functions as a possible determinant of the negative feedback control of the HPA axis in the general population. Also, instrumental disability, as an early indicator of cognitive problems, was associated with low cortisol levels after dexamethasone intake. Instrumental activities of daily living is a measure of the skills required to live independently and perform activities which are more complicated and higher-level tasks than in ADL such as shopping, managing finances, answering the telephone, preparing meals, or using transportation. Disability in iADL often reflects severity of chronic, disabling diseases and is associated to decreased quality of life and disturbed social life. Moreover, iADL is disturbed earlier than ADL in people with cognitive problems and it is used as a proxy of cognitive functioning. Further studies are needed to explore cognition in more detail in DST studies.

Symptoms of depression and major depressive disorders were not related to basal cortisol or suppression in linear models. Given the extensive evidence from clinical research on the relation between clinical depression and DST, there is little doubt that a certain percentage of depressed patients show non-suppression in the DST. Results of the studies in outpatients or in the general population, however, vary largely. In our study, we evaluated depressive symptoms in the general population, which may differ from the clinical populations in terms of severity of the disorder and socio-economical characteristics. Either, the depressive symptoms observed in this population have too little impact on HPA axis regulation, or the association is not uniform, i.e., depressive symptoms or disorders in some patients are related to a stronger and in others to less suppression of the HPA axis; this is possibly explained by subtypes of depression. Also, there is possibly a non-linear association between depression and the extremes of HPA axis functioning in the general population, which explains inconsistent results. This is supported by an association of depressive symptoms with non-suppression of the HPA axis by very low doses of dexamethasone in the absence of an association along the continuum of suppression. Such a non-linear pattern was not observed for MDD most likely this was due to the small sample of persons with clinical depression.
Similarly, previous research on anxiety disorders and the negative feedback of the HPA axis provided conflicting findings, such as non-suppression \(^{59, 60}\) and suppression \(^{61, 62}\) in different anxiety disorders. These inconsistencies in the literature could stem from different physiological features of individual anxiety disorders, chronicity of the disorders and comorbid psychiatric diseases, mainly depressive disorders \(^{62}\). Similar to depression, there was a non-linear association between anxiety and the negative feedback control of the HPA axis. In our study, we found that having an anxiety disorder was related to the non-suppression of the HPA axis after dexamethasone intake. HPA axis abnormalities are also seen in psychotic patients. A stronger suppression of the HPA axis after dexamethasone intake has been reported in patients with psychosis \(^{15}\). In the current study, we assessed psychotic experiences indicating a very mild form of psychosis. Research exploring the association between the HPA axis functioning and psychotic experiences is very limited and is restricted to assessments of cortisol levels or stress reactivity rather than the negative feedback control of the HPA axis \(^{63}\). In the current study, we did not detect any associations between psychotic experiences and cortisol levels after dexamethasone intake.

In the main analyses, we did not find any associations between psychotropic medications and the cortisol levels after dexamethasone intake. However, psychotropic medication use was related to the enhanced suppression of cortisol after dexamethasone intake. Both antidepressants and antipsychotics have been related to increased cortisol suppression after dexamethasone intake previously \(^{15, 64, 65}\) yet the underlying mechanisms remain unclear.

Our study had several strengths. First, this study had a large sample size, which is not common in studies using an interventional diagnostic test. Second, the population-based design of the current study increases the external validity of the results. Third, the very low-dose DST in this population-based study allowed us to detect subtle changes, which cannot be easily detected with a high dose DST in healthy people \(^7\). Fourth, the large sample size and extensive data collection of the study allowed us to evaluate several variables and determine which are the strongest predictors of the cortisol response after dexamethasone intake. Some limitations, however, should be considered. First, when used in outpatients or in the general population, the DST can suffer from the noncompliance to the dexamethasone protocol. Yet, noncompliance to dexamethasone intake was not very frequent in studies monitoring compliance, not even if participants with psychiatric disorders were included \(^5, 66\). Also, compliance in reporting the exact time of sampling could be a problem in the current study, as we did not use a saliva collection device recording time of sampling automatically. However, we instructed our participants only to return the saliva samples if they had taken the dexamethasone. We also emphasized the importance of recording the exact time of sampling. Participants were encouraged to report the true time of sampling to allow us to calculate the deviations from the planned times. As an indicator
of compliance, the time interval between the first and second cortisol sampling was used in the sensitivity analyses. We detected no difference between compliers and non-compliers (according to an arbitrary definition of compliance) and therefore presented the results of the whole group to minimize selection bias. However, our results might suffer from residual information bias if noncompliance was not measured well. Second, our results cannot be generalized to the clinical psychiatric populations as coping styles, perceived stress and SES determinants may differ largely between clinical cohorts and general population. Third, we did not evaluate trauma history or PTSD, which might be an important determinant to evaluate.16–19 However, the prevalence of PTSD is low. Moreover, obtaining a valid trauma history in older persons is challenging in the general population. Finally, testing non-linear associations with centiles increases the risk of type I error due to multiple testing. At the same time, it may lead to small subgroups, which reduce the statistical power. Indeed, the lack of an association between MDD and non-suppression can most likely be attributed to the small sample size of persons with MDD in the current study.

In this large population-based study, we found a consistent pattern of increased negative feedback control of cortisol using the very low-dose DST in females, smokers, less active persons, and those with low income and instrumental disability. In contrast, clinical and subclinical psychiatric and cognitive conditions were less important determinants than gender, low socioeconomic status and poor health. Prospective studies are needed to test to what extent increased suppression of cortisol is related to disease.
REFERENCES


SUPPLEMENTARY TEXT

Instructions of Saliva Sampling

Participants were instructed about the procedure. A detailed explanation of the reasoning of the test was given. Also, sampling times and time of dexamethasone intake were detailed and the importance of recording the exact time was emphasized. We informed participants about fasting and not brushing their teeth for 15 minutes before saliva sampling.

Participants were trained about using saliva-sampling devices, collecting saliva, storing them until the samples were collected. A dexamethasone pill was shown to the participants at the study center.

They were instructed to make an appointment as soon as they finish the procedure. Samples were either brought by participants or were collected by the study centre staff.

Figure A. Distribution of cortisol levels before dexamethasone intake (x-axis represents categories of cortisol values and y-axis represents percentage of people within the cortisol range)
The very low-dose dexamethasone suppression test in the general population

Figure B. Distribution of cortisol levels after dexamethasone intake (x-axis represents categories of cortisol values and y-axis represents percentage of people within the cortisol range)
Figure C. Effect of compliance to saliva cortisol sampling on the association of different determinants with cortisol suppression after dexamethasone intake. Regression coefficients and the confidence intervals of the associations between determinants and cortisol concentrations after dexamethasone intake in people with a time interval of 24 ± 1 hours (n=1632) between cortisol samplings and in people with a time interval of 24 ± 2 hours (n=1766). These estimates derived from the mutually adjusted model.
Table A. Relations of determinants with cortisol concentrations before dexamethasone intake

<table>
<thead>
<tr>
<th>Sociodemographic indicators</th>
<th>Cortisol concentration before dexamethasone intake</th>
<th>Mutually adjusted model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (0=male, 1=female)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low concentrated model</td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.04</td>
<td>0.004</td>
</tr>
<tr>
<td>Sex (0=male, 1=female)</td>
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<tr>
<td>Education</td>
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<tr>
<td>Low concentrated model</td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td>Low concentrated model</td>
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<td>-0.04</td>
</tr>
<tr>
<td>Intermediate concentrated model</td>
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<td>-0.05</td>
</tr>
<tr>
<td>High concentrated model</td>
<td>(reference)</td>
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</tr>
<tr>
<td>Net income (ranked 1 to 13)</td>
<td>-0.002</td>
<td>-0.01</td>
</tr>
<tr>
<td>Health &amp; lifestyle variables</td>
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</tr>
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<td>Mild/severe disability (0=no, 1=yes)</td>
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</tr>
<tr>
<td>Instrumental disability (0=no, 1=yes)</td>
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</tr>
<tr>
<td>Regular exercise (0=yes, 1=no)</td>
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</tr>
<tr>
<td>Smoking</td>
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<td></td>
</tr>
<tr>
<td>Never smoker (reference)</td>
<td>B</td>
<td>95% CI</td>
</tr>
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<td>Former smoker</td>
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<td>0.03</td>
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<tr>
<td>Current smoker</td>
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<td>-0.04</td>
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<td>Body mass index (kg/m²)</td>
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<td>0.03</td>
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<tr>
<td>Hypertension (0=no, 1=yes)</td>
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<td>-0.02</td>
</tr>
<tr>
<td>Psychiatric problems &amp; cognitive functions</td>
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<td></td>
</tr>
<tr>
<td>Depressive symptom score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinically relevant depressive symptoms (0=no, 1=yes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major depressive disorder (0=no, 1=yes)</td>
<td>0.04</td>
<td>0.18</td>
</tr>
<tr>
<td>Anxiety disorders (0=no, 1=yes)</td>
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<td>-0.02</td>
</tr>
<tr>
<td>Mini Mental State Examination score</td>
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<td>0.03</td>
</tr>
<tr>
<td>Cognitive impairment (0=no, 1=yes)</td>
<td>-0.06</td>
<td>-0.26</td>
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<tr>
<td>The g-factor of the cognitive tests</td>
<td>0.06</td>
<td>0.04</td>
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<td>Psychotic experiences (CAPE-positive items)</td>
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<td></td>
</tr>
<tr>
<td>Psychotropic medications (0=no, 1=yes)</td>
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<td>-0.08</td>
</tr>
<tr>
<td>Sampling variables</td>
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<tr>
<td>Day of the sampling (0=weekend, 1=weekday)</td>
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<td></td>
</tr>
<tr>
<td>Time of the sampling</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: β, standardized beta; B, unstandardized beta; CI, Confidence Interval of the unstandardized beta; MMSE, Mini Mental State Examination; CAPE, The Community Assessment of Psychic Experience
**Table B.** Relations of determinants with cortisol suppression categories

<table>
<thead>
<tr>
<th></th>
<th>Non-suppression (n=182)</th>
<th></th>
<th></th>
<th>Enhanced suppression (n=183)</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% Cl</td>
<td>p</td>
<td>OR</td>
<td>95% Cl</td>
<td>p</td>
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<td>Depressive symptom score</td>
<td>1.02</td>
<td>1.00; 1.05</td>
<td>.03</td>
<td>1.002</td>
<td>0.98; 1.02</td>
<td>.85</td>
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<td>Clinically relevant depressive symptoms*</td>
<td>1.93</td>
<td>1.18; 3.17</td>
<td>.01</td>
<td>1.05</td>
<td>0.63; 1.74</td>
<td>.86</td>
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<tr>
<td>Major depressive disorder*</td>
<td>1.51</td>
<td>0.50; 4.52</td>
<td>.46</td>
<td>0.61</td>
<td>0.14; 2.68</td>
<td>.51</td>
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<tr>
<td>Anxiety disorders*</td>
<td>2.42</td>
<td>1.46; 4.00</td>
<td>.001</td>
<td>0.72</td>
<td>0.39; 1.34</td>
<td>.30</td>
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<tr>
<td>Mini Mental State Examination score</td>
<td>1.05</td>
<td>0.95; 1.17</td>
<td>.35</td>
<td>0.98</td>
<td>0.91; 1.05</td>
<td>.52</td>
</tr>
<tr>
<td>Cognitive impairment*</td>
<td>1.51</td>
<td>0.44; 5.16</td>
<td>.51</td>
<td>1.45</td>
<td>0.57; 3.67</td>
<td>.43</td>
</tr>
<tr>
<td>The g-factor of the cognitive tests</td>
<td>0.97</td>
<td>0.79; 1.19</td>
<td>.76</td>
<td>0.87</td>
<td>0.72; 1.04</td>
<td>.13</td>
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<tr>
<td>Psychotic experiences (CAPE-positive items) frequency score</td>
<td>1.16</td>
<td>0.19; 7.13</td>
<td>.87</td>
<td>1.59</td>
<td>0.28; 9.01</td>
<td>.60</td>
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<tr>
<td>Psychotropic medications*</td>
<td>1.55</td>
<td>0.99; 2.45</td>
<td>.06</td>
<td>2.08</td>
<td>1.43; 3.02</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*0=no, 1=yes
Chapter 2.3

Genome wide association identifies common variants at the SERPINA6/SERPINA1 locus influencing plasma cortisol and corticosteroid binding globulin.

Jennifer L Bolton, Caroline Hayward, Nese Direk, John G Lewis, et al.

ABSTRACT

Variation in plasma levels of cortisol, an essential hormone in the stress response, is associated in population-based studies with cardio-metabolic, inflammatory and neuro-cognitive traits and diseases. Heritability of plasma cortisol is estimated at 30 - 60% but no common genetic contribution has been identified. The CORtisol NEtwork (CORNET) consortium undertook genome-wide association meta-analysis for plasma cortisol in 12,597 Caucasian participants, replicated in 2,795 participants. The results indicate that <1% of variance in plasma cortisol is accounted for by genetic variation in a single region of chromosome 14. This locus spans SERPINA6, encoding corticosteroid binding globulin (CBG, the major cortisol-binding protein in plasma), and SERPINA1, encoding α1-antitrypsin (which inhibits cleavage of the reactive centre loop that releases cortisol from CBG). Three partially independent signals were identified within the region, represented by common SNPs; detailed biochemical investigation in a nested sub-cohort showed all these SNPs were associated with variation in total cortisol binding activity in plasma, but some variants influenced total CBG concentrations while the top hit (rs12589136) influenced the immunoreactivity of the reactive centre loop of CBG. Exome chip and 1000 Genomes imputation analysis of this locus in the CROATIA-Korcula cohort identified missense mutations in SERPINA6 and SERPINA1 that did not account for the effects of common variants. These findings reveal a novel common genetic source of variation in binding of cortisol by CBG, and reinforce the key role of CBG in determining plasma cortisol levels. In turn this genetic variation may contribute to cortisol-associated degenerative diseases.
INTRODUCTION

The adrenal steroid hormone cortisol plays a vital role in adaptation to environmental stress. In response to stressors such as starvation, infection or injury, cortisol secretion is elevated by activation of the HPA axis. Cortisol acts predominantly through glucocorticoid receptors to induce a wide range of physiological responses, including liberating fuel (by facilitating gluconeogenesis and lipolysis), maintaining cardiovascular homeostasis (by inducing sodium retention and vasoconstriction), altering mood and memory (in favour of focusing on ‘fight or flight’ responses), and acting as a ‘brake’ on the innate immune response (preventing bystander damage from unrestrained inflammation). Chronic elevations in cortisol, however, may be maladaptive, as exemplified in patients with tumours of the pituitary or adrenal gland causing Cushing’s syndrome; here, elevated plasma cortisol is responsible for obesity, type 2 diabetes, hypertension, dyslipidaemia, depression, memory loss, impaired wound healing, osteoporosis, myopathy, and many other features.

Epidemiological data suggest that subtle activation of the HPA axis associates with many of these traits within the population, in people who do not harbour the tumours which cause overt Cushing’s syndrome. In these studies higher plasma cortisol concentration, measured in the morning, provided a robust marker of the activation of the HPA axis which accompanies high blood pressure, hyperglycaemia and dyslipidaemia, age-associated cognitive dysfunction, and low mood. Conversely, lower cortisol associates with immunological abnormalities, post-traumatic stress disorder (PTSD), and obesity (the inverse association with obesity is likely due to increased metabolic clearance of cortisol and confounds the positive association of cortisol with other cardiovascular risk factors, explaining some inconsistencies in the associations of cortisol with ‘metabolic syndrome’). Mechanisms underlying these associations remain uncertain, with most investigators suggesting abnormal central control of the HPA axis. A high proportion of cortisol in plasma is protein bound, mostly to corticosteroid binding globulin (CBG). Although variations in total CBG concentrations have been associated with features of metabolic syndrome, this does not account entirely for associations of total plasma cortisol with other quantitative traits.

Morning plasma cortisol has a heritability of 30 – 60%. Identifying genetic variants which contribute to variation in morning cortisol values could provide key insights into the mechanism of HPA axis activation associated with common quantitative traits, and an opportunity to dissect causality using Mendelian randomisation. Attempts to identify these genetic variants to date have been limited to small candidate gene studies. We therefore established the CORtisol NEtwork (CORNET) consortium with the initial aim of identifying genetic determinants of inter-individual variation in HPA axis function.
RESULTS

Genome-wide Association Meta-analysis

We conducted a discovery meta-analysis of genome-wide association studies (GWAMA) of morning plasma cortisol levels, investigating ~2.5 M SNPs in 12,597 men and women, aged 14–102 years, of European origin (Table S1 for participant characteristics). There was very little inflation of test statistics ($\lambda_{GC} = 1.005$, Table S2). The $-\log_{10}(p)$ values by chromosome for age- and sex-adjusted cortisol $z$-scores are shown in Figure 1A. A quantile–quantile plot (Figure 1B) showed marked departure from the null for SNPs with low $p$ values, listed in Table S3. Analysis of data for men and women separately showed no sex-specific effects (data not shown). The results were similar between all multivariable adjusted models, and whether or not time of sampling was included as a covariate. The results reported are therefore adjusted only for age and sex.

Figure 1. Meta-analysis of genome wide association studies for morning plasma cortisol
A Manhattan plot of $-\log_{10}(p)$ values by chromosome. The red horizontal line indicates genome-wide significance ($p < 5\times10^{-8}$) and the blue horizontal line indicates moderate significance ($p < 5\times10^{-5}$). The lead SNP rs12589136 (chr14:94,793,686; b37) in red is genome-wide significant. SNPs within ± 50 kb of cortisol-related candidate genes (listed in Table S6) are highlighted in colours.
B Quantile-quantile plot of $-\log_{10}(p)$, comparing the distribution of observed $-\log_{10}(p)$-values and that expected by chance

There was strong evidence for associations between plasma cortisol and genetic variation found at chromosome 14q32. In an additive genetic model, the lead SNP rs12589136 reported a per minor allele effect of 0.10 cortisol $z$-score (95% CI 0.07, 0.13; $p = 4.0\times10^{-12}$ after genomic control). The effect allele frequency was 0.22 and this variation explained 0.13% of the morning plasma cortisol variance. A forest plot showed consistent directional effects in all studies, with the T allele at rs12589136 associated with higher morning plasma cortisol (Figure 2). Only minimal heterogeneity was observed between studies ($I^2 = 0.18$).
A recombination boundary containing SERPINA6 and SERPINA1 was found to contain all variants at this locus contributing to association with plasma cortisol (Figure 3). A clumping procedure20 identified rs12589136 (4 kb upstream of SERPINA6), rs11621961 (1 kb downstream of SERPINA6) and rs2749527 (30 kb upstream of SERPINA6) as markers representing genome-wide significant signals in this region. Individually, the beta (for effect on cortisol z-score, 95% CI) for minor (all T) alleles at rs12589136, rs2749527, and rs11621961 were 0.10 (0.07, 0.13; \( p = 3.3 \times 10^{-12} \)), −0.08 (−0.11, −0.06; \( p = 5.2 \times 10^{-11} \)), and −0.08 (−0.10, −0.05; \( p = 4.0 \times 10^{-8} \)); joint analysis showed these SNPs have partially independent effects, with beta (95% CI) 0.07 (0.04, 0.10; \( p = 3.1 \times 10^{-5} \)), −0.04 (−0.07, −0.01; \( p = 0.012 \)), and −0.03 (−0.07,0.00; \( p = 0.037 \)), respectively.

The SERPINA6 gene encodes corticosteroid binding globulin (CBG). The neighbouring (upstream) gene, SERPINA1, encodes α1-antitrypsin, the inhibitor of neutrophil elastase which cleaves and inactivates CBG21.
Figure 3. Regional associations surrounding lead SNP rs12589136 in genome-wide meta-analysis of morning plasma cortisol. Regional plot shows $-\log_{10}(p)$ values of all SNPs, and degree of correlation between all SNPs and lead SNP rs12589136. SNPs with lower $P$ values span SERPINA6 and SERPINA1 genes within a recombination boundary.

Conditional Analysis

A quantile-quantile plot after removal of the SERPINA6/SERPINA1 region (chr14: 94,768,859–94,843,565; Genome Reference Consortium build 37) showed no evident inflation of test statistics (not shown). Conditional analyses adjusting for each of the partially independent genome-wide significant variants (rs12589136, rs11621961, rs2749527) in a subset of the meta-analysis population reduced the significance of all other SNPs to $p > 1 \times 10^{-5}$ for an association with plasma cortisol.

Gene-centric Associations

We used a gene-centric approach to analyse the combined effect of all SNPs within a gene, rather than individual SNP associations, using VEGAS$^{22}$. This produced a gene-based test statistic from meta-analysis results, allowing identification of genes containing multiple SNPs that individually did not reach genome-wide significance (Table S4). Only SERPINA6 and SERPINA1 were identified as gene-wide significant ($p < 3 \times 10^{-6}$); both included rs12589136 in the gene boundary.
Candidate Gene Analysis
A list of 61 candidate genes thought likely to influence plasma cortisol was collated by a panel of experts. A Manhattan plot of the $-\log_{10}(p)$ values highlighted for SNPs in these candidate genes showed that only \textit{SERPINA6} reached genome-wide significance (Figure 1A). Using gene-based p-values from VEGAS, after adjusting for multiple testing, only \textit{SERPINA6} was associated with plasma cortisol (Table S5). \textit{SERPINA1} was not included in the candidate gene list.

Replication
In 2,795 participants in additional cohort studies, the association with plasma cortisol was replicated for the lead SNP rs12589136 ($p=0.0002$), rs11621961 ($p=0.003$) and rs2749529 (used as proxy for rs2749527, $p=0.019$) (Table 1; Table S1 for participant characteristics; Table S6 for results in each cohort).

Functional Consequences of Genetic Variation in the SERPINA6/SERPINA1 Region
We explored the associations between rs12589136, rs11621961, rs2749527 with cortisol and CBG phenotypes in more detail in 316 subjects from the CROATIA-Korcula cohort (Table 2). Together these three SNPs explained 0.54% of the variance in total plasma cortisol in CROATIA-Korcula. However, there were distinct patterns of association of ‘high cortisol’ alleles with CBG. After adjusting for age and sex, although all three variants were associated with differences in total cortisol binding activity, measured by the binding of $[^{3}\text{H}]$-cortisol, there were different associations with CBG immunoreactivity. The T allele at rs2749527 was associated with higher ‘total’ CBG concentration by radioimmunoassay, and there were similar, but weaker associations of total CBG immunoreactivity with variation at rs11621961. Differences in calculated free plasma cortisol reflected these differences in total CBG immunoreactivity, which is used in the calculation of free cortisol. In contrast, however, the minor (T) allele at rs12589136 was not associated with ‘total’ CBG immunoreactivity but was strongly associated with the proportion of CBG bound by a monoclonal antibody against an epitope in the reactive centre loop of CBG$^{23}$. None of these SNPs representing signals in the \textit{SERPINA6/SERPINA1} region was associated with \textit{a1}-antitrypsin concentrations in blood. However, \textit{a1}-antitrypsin levels were negatively correlated with plasma total cortisol (beta $-0.17$ (95% CI $-0.28$, $-0.06$); $p=0.002$) and calculated free cortisol (beta $-0.13$ (95% CI $-0.24$, $-0.02$); $p=0.021$), although they did not correlate with ratio of intact/total CBG (beta 0.02 (95% CI $-0.09$, 0.13; $p=0.715$).
### Table 1. Association with morning plasma cortisol of SNPs representing loci in the SERPINA6/SERPINA1 region from meta-analyses of discovery genome-wide association studies and of replication studies

<table>
<thead>
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<th>SNP ID</th>
<th>Chr</th>
<th>Number of supporting SNPs</th>
<th>Position (b37)</th>
<th>Alleles</th>
<th>GWAMA (n=12,597)</th>
<th>REPLICATION (n=2,795)</th>
<th>p*</th>
<th>EAF</th>
<th>Effects</th>
<th>Beta (95% CI)</th>
<th>p*</th>
<th>EAF</th>
<th>Effects</th>
<th>Beta (95% CI)</th>
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<tbody>
<tr>
<td>rs12589136</td>
<td>14</td>
<td>30</td>
<td>94,793,686</td>
<td>T/G</td>
<td>++++++++++++</td>
<td>0.10 (0.07,0.13)</td>
<td>3.3x10^{-12}</td>
<td>0.21</td>
<td>+++</td>
<td>0.12 (0.06,0.18)</td>
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<td>rs2749527</td>
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<td>17</td>
<td>94,827,068</td>
<td>T/C</td>
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<td>5.2x10^{-11}</td>
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<td>n/a</td>
<td>n/a</td>
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<td>rs2749529</td>
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<td>n/a</td>
<td>94,820,459</td>
<td>T/A</td>
<td>++++++++++++</td>
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<td>2.6x10^{-6}</td>
<td>0.45</td>
<td>+++</td>
<td>0.06 (0.01,0.11)</td>
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<td>rs11621961</td>
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<td>0</td>
<td>94,769,476</td>
<td>T/C</td>
<td>---------?----*</td>
<td>-0.08 (-0.10,-0.05)</td>
<td>4.0x10^{-8}</td>
<td>0.37</td>
<td>--</td>
<td>-0.08 (-0.14,-0.03)</td>
<td>0.003</td>
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</table>

*adjusted for age and sex.

rs2749527 was replaced with rs2749529 (r² = 0.905, D’ = 1.0) as rs2749527 failed manufacture for replication. LD patterns (from SNAP HapMap CEU build 22): rs11621961-rs12589136 (r² = 0.131), rs11621961-rs2749529 (r² = 0.260), rs12589136-rs2749529 (r² = 0.291), rs11621961-rs2749527 (r² = 0.255). Independent SNPs were defined by PLINK using the clumping function (within 500kb, LD r² > 0.2, p value < 5x10^{-5}) rs12589136-rs2749529 (r² = 0.50).
We investigated exome chip data for this locus in all CROATIA-Korcula participants to identify non-synonymous variants in the SERPINA6/SERPINA1 region associated with plasma cortisol. From 34 variants on the exome chip in this region (chr14:94,770,585–94,857,029, build 37), 9 were polymorphic in this sample (Table S7) but only two were associated with plasma cortisol: rs113418909 in SERPINA6 (Leu115His in CBG, previously reported as the Leuven mutation associated with low total cortisol); and rs28931570 in SERPINA1 (Arg63Cys in α1-antitrypsin, not recognised as a disease-causing variant).

We also analysed 735 additional SNPs in the same region imputed from 1000 Genomes data in all CROATIA-Korcula participants but did not find any additional SNPs associated with plasma cortisol, using a $p$-value threshold of $0.05/735 = 7 \times 10^{-5}$. The two rare variants rs113418909 and rs28931570 were in perfect linkage disequilibrium amongst participants with detailed biochemical phenotyping performed, so results are reported for rs113418909 only (Table 2). Prevalence of the Leuven variant in CROATIA-Korcula was higher than expected (MAF = 0.017, compared with MAF = 0.0046 in dbSNP). After adjusting for age, sex, and accounting for kinship, participants who were heterozygote for the Leuven variant had lower total cortisol and markedly lower total cortisol binding activity, but normal CBG immunoreactivity (Table 2).

After removing subjects with the rare Leuven variant (ie. heterozygotes), rs12589136 remained associated with total cortisol (beta 0.15, 95% CI 0.04, 0.26; $p = 0.009$), calculated free cortisol (0.17, 95% CI 0.02, 0.32; $p = 0.031$), and the proportion of CBG bound by the reactive centre loop antibody ($-0.45$, 95% CI $-0.60$–$-0.29$; $p = 2.8 \times 10^{-8}$).

### Table 2. Functional consequences of variants in loci significantly associated with morning plasma cortisol in GWAMA, and of the Leuven variant, in CROATIA-Korcula

<table>
<thead>
<tr>
<th></th>
<th>rs11621961</th>
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<tbody>
<tr>
<td></td>
<td>TT</td>
<td>CT</td>
<td>CC</td>
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</tr>
<tr>
<td>Total cortisol$^1$</td>
<td>655 (618,694) [129]</td>
<td>664 (644,685) [411]</td>
<td>675 (654,696) [357]</td>
<td>-0.05 (-0.14,0.05)</td>
<td>0.305</td>
<td></td>
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</tr>
<tr>
<td>Calculated free cortisol</td>
<td>42 (35.0,51) [56]</td>
<td>47 (42,52) [126]</td>
<td>50 (46,55) [136]</td>
<td>-0.14 (-0.28,0.00)</td>
<td>0.049</td>
<td></td>
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</tr>
<tr>
<td>Measured free cortisol$^2$</td>
<td>14 (11,17) [27]</td>
<td>14 (11,16) [70]</td>
<td>13 (10,16) [67]</td>
<td>0.01 (-0.2,0.22)</td>
<td>0.933</td>
<td></td>
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</tr>
<tr>
<td>Total CBG</td>
<td>0.90 (0.82,0.99) [56]</td>
<td>0.90 (0.85,0.94) [70]</td>
<td>0.96 (0.92,1.01) [136]</td>
<td>-0.12 (-0.26,0.02)</td>
<td>0.103</td>
<td></td>
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</tr>
<tr>
<td>Intact/total CBG</td>
<td>0.76 (0.71,0.80) [56]</td>
<td>0.77 (0.74,0.79) [126]</td>
<td>0.76 (0.73,0.78) [136]</td>
<td>0.01 (-0.14,0.15)</td>
<td>0.940</td>
<td></td>
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</tr>
<tr>
<td>α1-antitrypsin</td>
<td>2.74 (2.37,3.16) [56]</td>
<td>2.74 (2.51,2.99) [124]</td>
<td>2.66 (2.43,2.91) [134]</td>
<td>0.02 (-0.14,0.17)</td>
<td>0.840</td>
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### rs12589136

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<tr>
<td>Total cortisol¹</td>
<td>706 (656,760)</td>
<td>681 (657,706)</td>
<td>655 (638,673)</td>
<td>0.13 (0.02,0.24)</td>
</tr>
<tr>
<td></td>
<td>[53]</td>
<td>[302]</td>
<td>[540]</td>
<td></td>
</tr>
<tr>
<td>Calculated free cortisol</td>
<td>47 (41,54)</td>
<td>54 (45,65)</td>
<td>46 (42,50)</td>
<td>0.13 (-0.01,0.26)</td>
</tr>
<tr>
<td></td>
<td>[52]</td>
<td>[47]</td>
<td>[217]</td>
<td></td>
</tr>
<tr>
<td>Measured free cortisol²</td>
<td>13 (11,16)</td>
<td>n/a</td>
<td>13 (12,15)</td>
<td>0.03 (-0.15,0.2)</td>
</tr>
<tr>
<td></td>
<td>[39]</td>
<td></td>
<td>[125]</td>
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</tr>
<tr>
<td>Total CBG</td>
<td>1.01 (0.95,1.07)</td>
<td>0.91 (0.82,1.02)</td>
<td>0.91 (0.87,0.95)</td>
<td>0.06 (-0.07,0.20)</td>
</tr>
<tr>
<td></td>
<td>[52]</td>
<td>[47]</td>
<td>[217]</td>
<td></td>
</tr>
<tr>
<td>Intact/total CBG</td>
<td>0.68 (0.64,0.71)</td>
<td>0.69 (0.65,0.73)</td>
<td>0.80 (0.78,0.82)</td>
<td>-0.49 (-0.64,-0.34) 9.4x10⁻¹¹</td>
</tr>
<tr>
<td></td>
<td>[52]</td>
<td>[47]</td>
<td>[216]</td>
<td></td>
</tr>
<tr>
<td>α1-antitrypsin</td>
<td>2.68 (2.36,3.05)</td>
<td>2.77 (2.43,3.16)</td>
<td>2.69 (2.50,2.90)</td>
<td>0.06 (-0.09,0.22)</td>
</tr>
<tr>
<td></td>
<td>[51]</td>
<td>[47]</td>
<td>[214]</td>
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### rs2749527

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<th>CT</th>
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<tbody>
<tr>
<td>Total cortisol¹</td>
<td>659 (635,684)</td>
<td>668 (649,687)</td>
<td>674 (643,707)</td>
<td>0.03 (-0.06,0.12)</td>
</tr>
<tr>
<td></td>
<td>[251]</td>
<td>[431]</td>
<td>[212]</td>
<td></td>
</tr>
<tr>
<td>Calculated free cortisol</td>
<td>49 (43,55)</td>
<td>45 (41,50)</td>
<td>48 (42,55)</td>
<td>0.02 (-0.11,0.16)</td>
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<tr>
<td></td>
<td>[102]</td>
<td>[123]</td>
<td>[92]</td>
<td></td>
</tr>
<tr>
<td>Measured free cortisol²</td>
<td>16 (14,19)</td>
<td>11 (8,15)</td>
<td>13 (11,15)</td>
<td>-0.11 (-0.29,0.07)</td>
</tr>
<tr>
<td></td>
<td>[60]</td>
<td>[51]</td>
<td>[53]</td>
<td></td>
</tr>
<tr>
<td>Total CBG</td>
<td>0.87 (0.82,0.92)</td>
<td>0.92 (0.87,0.98)</td>
<td>1.00 (0.96,1.05)</td>
<td>0.17 (0.03,0.30)</td>
</tr>
<tr>
<td></td>
<td>[102]</td>
<td>[123]</td>
<td>[92]</td>
<td></td>
</tr>
<tr>
<td>Intact/total CBG</td>
<td>0.76 (0.73,0.79)</td>
<td>0.76 (0.74,0.79)</td>
<td>0.76 (0.73,0.79)</td>
<td>-0.02 (-0.16,0.13)</td>
</tr>
<tr>
<td></td>
<td>[102]</td>
<td>[122]</td>
<td>[92]</td>
<td></td>
</tr>
<tr>
<td>α1-antitrypsin</td>
<td>2.58 (2.31,2.88)</td>
<td>2.86 (2.62,3.12)</td>
<td>2.65 (2.38,2.94)</td>
<td>0.08 (-0.06,0.23)</td>
</tr>
<tr>
<td></td>
<td>[101]</td>
<td>[121]</td>
<td>[91]</td>
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### rs113418909 (Leuven)

<table>
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<tr>
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<th>AT</th>
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<tbody>
<tr>
<td>Total cortisol</td>
<td>673 (658,688)</td>
<td>562 (500,631)</td>
<td>-0.63 (-1.01,-0.25)</td>
</tr>
<tr>
<td></td>
<td>[792]</td>
<td>[28]</td>
<td></td>
</tr>
<tr>
<td>Calculated free cortisol</td>
<td>48 (45,52)</td>
<td>38 (27,52)</td>
<td>-0.43 (-1.06,0.20)</td>
</tr>
<tr>
<td></td>
<td>[280]</td>
<td>[9]</td>
<td></td>
</tr>
<tr>
<td>Measured free cortisol²</td>
<td>14 (12,15)</td>
<td>13 (8,20)</td>
<td>-0.04 (-0.92,0.83)</td>
</tr>
<tr>
<td></td>
<td>[147]</td>
<td>[5]</td>
<td></td>
</tr>
<tr>
<td>Total CBG</td>
<td>0.93 (0.90,0.96)</td>
<td>0.94 (0.82,1.07)</td>
<td>0.05 (-0.58,0.68)</td>
</tr>
<tr>
<td></td>
<td>[280]</td>
<td>[9]</td>
<td></td>
</tr>
<tr>
<td>Intact/total CBG</td>
<td>0.76 (0.74,0.78)</td>
<td>0.82 (0.78,0.87)</td>
<td>0.51 (-0.18,1.19)</td>
</tr>
<tr>
<td></td>
<td>[279]</td>
<td>[9]</td>
<td></td>
</tr>
<tr>
<td>α1-antitrypsin</td>
<td>2.67 (2.51,2.84)</td>
<td>2.40 (1.91,3.03)</td>
<td>-0.12 (-0.83,0.58)</td>
</tr>
<tr>
<td></td>
<td>[277]</td>
<td>[9]</td>
<td></td>
</tr>
</tbody>
</table>

¹Data for total cortisol is from the whole Croatia-Korcula sample, n=898
²Samples selected for measured free cortisol assay were age and sex matched homozygotes at rs12589136
³adjusted for age, sex, and first three principal components, used kinship matrix derived from GWAS data
Data are geometric mean (95% CI) [n]. Cortisol values are nmol/L, CBG micromol/L and α1-antitrypsin g/L
DISCUSSION

These results clearly attribute inter-individual differences in morning plasma cortisol amongst Europeans to genetic variation within a region on chromosome 14 containing the SERPINA6 and SERPINA1 genes. The association of this region with plasma cortisol was consistent across multiple cohorts and was observed not only in genome-wide meta-analysis of individual SNPs, but also in gene-based hypothesis-free analysis, and in a candidate gene analysis. Investigation of the functional consequences of genetic variation in this region in a genetic isolate population in Croatia indicates that the effects of variation at SERPINA6 and SERPINA1 on plasma cortisol are likely to be mediated through alterations in total cortisol binding by corticosteroid binding globulin (CBG). In part, this is determined by differences in total CBG concentrations, and in part in association with a previously unrecognised variability in the immunoreactivity of the reactive centre loop of CBG. Since the process of CBG cleavage by neutrophil elastase and resultant reconfiguration of the reactive centre loop is considered important in the release of bioavailable cortisol within target tissues\textsuperscript{21,26}, this finding provides a novel insight into a biological pathway controlling cortisol action.

The diverse actions of cortisol, and the striking clinical consequences of glucocorticoid excess or deficiency, have led many investigators to propose a central role for variations in cortisol levels in determining common quantitative traits. However, cortisol has not been measured as widely in epidemiological cohort studies as many other phenotypes. This may reflect the perceived difficulty of obtaining samples at a fixed time of day and in un-stressed conditions, to avoid confounding effects. The CORNET consortium had to decline samples from many cohorts in which time of sampling was inadequately controlled, and even then there was high variability in plasma cortisol. Thus, although the variants we identified in the SERPINA6/SERPINA1 region of chromosome 14 accounted for <1% of the variance in plasma cortisol, this signal may be obscured by substantial unmeasured confounding and measurement error and may comprise a considerable component of the estimated 30–60% heritability of plasma cortisol\textsuperscript{16–18}. We identified 3 SNPs with partially independent effects on plasma cortisol. There may be a small degree of linkage disequilibrium between these SNPs, but they also show different associations with CBG biochemistry, suggesting that they represent independent effects. None of these 3 SNPs appears directly to affect CBG function; although rs12589136 is close to a consensus estrogen response element, there was no gender difference in its association with plasma cortisol.

Previous investigations of the genetic determinants of plasma cortisol\textsuperscript{18} have been under-powered candidate gene studies, including some which included a tandem repeat in intron 1 of SERPINA6\textsuperscript{22,28}. Interestingly, many of the genetic variants previously associated with
cortisol, eg for glucocorticoid\textsuperscript{1} and mineralocorticoid\textsuperscript{29, 30} receptors, showed no signal whatsoever in the adequately powered GWAMA and candidate gene analysis conducted here.

Rare mutations in \textit{SERPINA6} have been described which cause absent CBG protein or, more often, reduced affinity of CBG for cortisol\textsuperscript{31-34}. Affected individuals have low total plasma cortisol but normal free plasma cortisol. However, they also have abnormal pulsatility of plasma cortisol, and non-specific symptoms including fatigue which are unresponsive to cortisol supplementation; features which have been attributed to abnormal function of CBG in delivering cortisol to target tissues, including in brain regions involved in negative feedback regulation of the HPA axis\textsuperscript{21}. Although one of these mutations, A51V, has been found to be surprisingly prevalent (MAF>3\%) amongst Chinese subjects\textsuperscript{34}, it has not been found in non-Asian populations and we did not find Caucasians carrying this mutation when tested by exome chip analysis. In cohort studies, plasma CBG concentrations have been associated with features of the metabolic syndrome\textsuperscript{12, 13, 15} and one previous candidate gene study with >900 participants showed that SNPs in \textit{SERPINA6}, including some identified as being associated with plasma cortisol in this GWAMA, were predictive of somatic symptoms\textsuperscript{35}. We found evidence that genetic variation in the \textit{SERPINA6/SERPINA1} region influences total plasma cortisol not only through changes in total CBG concentrations, but also in association with alterations in the immunoreactivity of the reactive centre loop of the CBG protein.

Cleavage of the reactive centre loop (RCL) of CBG by neutrophil elastase and inhibition of elastase activity by \(\alpha\)1-antitrypsin has been recognised for more than 20 years\textsuperscript{26}. However, the recent development of monoclonal antibodies which recognise the intact RCL of CBG has allowed this process to be studied \textit{in vivo} for the first time\textsuperscript{23}. Using these tools in samples from Croatia-Korcula has provided the novel insight that immunoreactivity of the RCL of CBG is variable in the population, and further that this is explained in part by genetic variations in the \textit{SERPINA6/SERPINA1} region. It remains to be determined whether this difference in immunoreactivity of the RCL represents altered susceptibility to CBG cleavage. We show that a common variant (rs12589136) associated with impaired RCL antibody binding was associated with higher total plasma cortisol and higher cortisol binding activity. These observations are inconsistent with the interpretation that impaired RCL antibody binding represents enhanced RCL cleavage\textsuperscript{23}, given that cleaved CBG has a lower affinity than intact CBG for cortisol binding\textsuperscript{36}. Alternatively, the altered immunoreactivity of the RCL epitope may represent resistance to cleavage and hence enhanced cortisol binding. It is possible that the genetically determined difference in the RCL epitope of CBG is associated with impaired negative feedback of the HPA axis due to reduced tissue delivery of cortisol by CBG, analogous with findings in CBG knockout mice\textsuperscript{37}. Although we could
not confirm associated elevation in free plasma cortisol concentrations, these measure-
ments are notoriously unreliable, for example being similarly unhelpful in dissecting the
consequences of CBG deficiency described above.

We found further evidence for the importance of CBG using exome chips in the genetic
isolate population of Korcula in Croatia, where we discovered an unusually high prevalence
of heterozygotes for the Leuven mutation in \textit{SERPINA6}^{24}. These individuals have lower total
plasma cortisol despite normal total CBG concentrations, and we confirmed substantial
reductions in total cortisol binding activity, without any difference in RCL antibody binding.
The presence of the Leuven variant, however, did not account for the association of the top
hit SNPs identified by GWAMA with plasma cortisol or CBG RCL antibody binding.

It is possible that a combination of alterations in CBG substrate as well as in neutrophil
elastase level and/or activity may determine cleavage of CBG and tissue delivery of corti-
sol, especially in local sites of inflammation\textsuperscript{21}. Intriguingly, we found inverse associations
between levels of α1-antitrypsin, the inhibitor of neutrophil elastase, and plasma cortisol
concentrations, consistent with instability of CBG resulting in HPA axis activation as pro-
posed above; however, we could not identify a genetic influence on this relationship, or
confirm its association with CBG RCL immunoreactivity. Specifically, we did not identify
independent signals for \textit{SERPINA6} and \textit{SERPINA1} in conditional analysis, and the rare vari-
ant Leuven mutation was in linkage disequilibrium with the only rare variant we identified
in \textit{SERPINA1}. Recent studies have identified variants in \textit{SERPINA1} that are associated with
coronary artery calcification\textsuperscript{38} and serum lipid profile\textsuperscript{39}, the latter represented by rs1303
which is in linkage disequilibrium with the top hit rs12589136 identified by GWAMA
($r^2 = 0.35$). These findings are consistent with variation in the \textit{SERPINA6/SERPINA1} locus
affecting downstream actions of cortisol, but it remains unclear if an interaction exists
between the variants at these two genes. Mutations in \textit{SERPINA1} cause the syndrome of
α1-antitrypsin deficiency, but we are not aware of any investigations of CBG or cortisol in
these patients, and their HPA axis may be disturbed anyway by un-restrained neutrophil-
mediated tissue damage. Rare variants in \textit{SERPINA1} (notably rs112635299) have been
associated with α 1-globulin plasma protein levels, of which α1-antitrypsin is a major
constituent, using GWAS with 1000 Genomes imputation\textsuperscript{40}. However, in the CROATIA-
Korcula cohort neither rs1303 (Table S7) nor SNPs imputed from 1000 Genomes (including
rs112635299) were associated with plasma cortisol. More detailed phenotyping amongst
participants with contrasting genotypes at the \textit{SERPINA6/SERPINA1} region will be required
to clarify the basis for altered interaction between the two gene products.

These findings emphasise the biological importance of plasma protein binding for steroid
hormones, and are analogous to recent findings that a common variant in sex hormone
Chapter 2.3

Binding globulin contributes to variation in total testosterone levels\(^{31}\), \(^{41}\). Given the consequences of altered binding protein function for steroid volume of distribution and clearance, and documented effects on HPA axis function\(^{31}\), this is an important finding of itself. However, potentially of greater importance is the novel observation that a key protein domain of CBG, the reactive centre loop, is subject to inter-individual differences which are influenced by genetic variation and may constitute a novel influence on tissue steroid action.

**MATERIALS AND METHODS**

**Gene Discovery**

We performed a meta-analysis of genome-wide association studies of morning plasma cortisol in 12,597 subjects from 11 western European population-based cohorts: CROATIA-Vis (n = 885), CROATIA-Korcula (n = 898), CROATIA-Split (n = 493), ORCADES (n = 886), Rotterdam Study (n = 2945), NFBC1966 (n = 1195), Helsinki Birth Cohort Study 1934–44 (n = 451), ALSPAC (n = 1567), InChianti (n = 1207), PREVEND (n = 1151), and PIVUS (n = 919). Replication was tested in 2,795 subjects from three independent cohorts: Raine Study (n = 797), ET2DS (n = 1,069), and MrOS-Sweden (n = 929). Cortisol was measured by immunoassay in blood samples collected from study participants between 0700 and 1100 h. Inclusion criteria were adults aged 17 years or older from Caucasian populations; exclusion criteria were current glucocorticoid use, pregnant or breast feeding women, and twins (exclusion of one). Characteristics of the study populations are presented in Table S1 and details of each cohort are provided in Text S1. All participants provided written informed consent and studies were approved by local Research Ethics Committees and/or Institutional Review Boards.

**Association Analysis with Morning Plasma Cortisol**

Each study performed single marker association tests, and study-specific linear regression models which used z-scores of log-transformed cortisol, additive SNP effects, and were adjusted for age and sex (model 1); age, sex, and smoking (model 2); or age, sex, smoking and body mass index (model 3). Imputation of the gene-chip results used the HapMap CEU population, build 36. In cohorts with consanguineous populations (ORCADES and Croatia), adjustments for principal components in kinship matrices were performed using ProbABEL; for other cohorts, Identity-By-Descent coefficients were calculated using PLINK and related participants excluded. In the majority of cohorts, participants were only included if blood samples had been obtained within a 60 minute time interval, when variations in time of sampling were ignored. In a subset of cohorts, samples were obtained over a wider time interval (but always in the morning before 11:00 h) and time of blood sampling
recorded; for these cohorts, three further models were run as above but also including time of sampling, calculated as minutes from first sampling time, as an additional covariate. Quality control was carried out on the imputed genome-wide data for all 11 studies prior to meta-analysis; this excluded all samples with a minor allele frequency (MAF) <2%, call rate <95%, Hardy-Weinberg equilibrium (HWE) <1×10^{-8} and poor imputation quality (MACH R2_HAT< 0.30, IMPUTE PROPER_INFO< 0.60, BEAGLE INFO< 0.30, as appropriate). Quantile-quantile (Q-Q) plots and genomic control (lambda) were used to confirm quality control. Sex chromosomes were not analysed.

**Meta-analysis of Association Results**

We performed fixed effects meta-analysis, which used combined allelic effects weighted by the inverse of their variance for each of the models using the GWAMA program. This aligned all studies to the same reference allele at each SNP, thus avoiding strand errors, and excluded SNPs with obvious input errors (e.g. discrepancies in effect allele frequencies). The results from analysis with or without genomic control were nearly identical, as expected with GC = 1.005. The genome-wide significance threshold for the meta-analysis was \( p < 5 \times 10^{-8} \). Percentage variation of cortisol was calculated from meta-analysis results as \( (2 \times \text{effect allele frequency}) \times (1 - \text{effect allele frequency}) \times (\beta^2 / \sigma^2) \). A regional plot was generated using LocusZoom, and heat map using snp.plotter in R version 2.15.2. Joint analysis of meta-analysis was performed with the GCTA program.

**Clumping Analysis**

To detect independent top SNPs on the basis of empirical estimates of linkage disequilibrium between the SNPs, we used the clumping function as implemented in PLINK. All the SNPs with a \( p \)-value<5×10^{-5} in meta-analysis were used for clumping. We grouped the SNPs within 500 kb of the index SNP that have \( r^2 > 0.2 \) with the index SNP.

**Replication Genotyping and Analysis**

Genes identified in the meta-analysis were evaluated in the Raine Study, MrOS-Sweden, and Edinburgh type 2 Diabetes Study (ET2DS). Raine Study and MrOS-Sweden had GWAS data so we extracted the replication SNP results, and ET2DS was genotyped at the Wellcome Trust Clinical Research Facility Genetics Core Laboratory in Edinburgh using the OpenArray genotyping platform. rs2749527 failed manufacture for the OpenArray, and was replaced with rs2749529 (\( r^2 = 0.905, D’ = 1.0 \)) in all replication cohorts. Genotypic association analysis in these studies followed the same methods as those described above for the discovery meta-analysis, adjusting for age and sex.
Conditional & sex-specific analysis

We performed meta-analyses of sex-specific GWAS and conditional GWAS in a subset of populations, using single marker association tests of z-scores of log-transformed cortisol. For the sex-specific analysis, each study adjusted for age in both men (n=3,546) and women (n=5,956). For the conditional analysis, each study adjusted for age, sex, and each of the SERPINA6 loci SNPs rs12589136 (n=9,308), rs11621961 (n=7,687), and rs2749527 (n=9,307) individually. We then did fixed effects meta-analysis using GWAMA program.

Gene-based Analysis

We used the Versatile Gene-based Association Study (VEGAS) program to perform gene-centric analysis. This used individual SNP \( p \)-values derived from the meta-analysis results to compute a gene-based \( p \)-value. We used two methods: all SNPs within a gene, or a subset of the 10% most significant SNPs in each gene boundary. VEGAS accounted for linkage disequilibrium between SNPs using the HapMap phase 2 population (CEU). SNPs were assigned to \( \sim \)18,000 genes based on positions in build 36 (hg18), with gene boundaries of \( \pm50 \) kb of the UTR. Bonferroni corrected threshold for gene-wide significance was \( 3 \times 10^{-6} \). The overlap of SNPs included in the gene boundaries in our results indicates this is likely to be an overly conservative correction factor.

A list of 61 candidate genes with known biological function in the regulation of cortisol was compiled by a panel of experts in the field. All SNPs within and \( \pm50 \) kb of these genes were examined in the GWAMA results, and gene-based \( p \) values were inspected in VEGAS results.

Exome Chip and 1000 Genomes Imputed Data Analysis

Genotypes for the SERPINA6/SERPINA1 gene region (chr14:94,770,585–94,857,029, build 37) in the CROATIA-Korcula samples (n=898) were extracted from an Illumina Exome Chip v1 analysis. Genotypes were called in GenomeStudio (Illumina) using the CHARGE Consortium joint calling cluster file (http://www.chargeconsortium.com/main/exomechip). 1000 Genomes imputation was performed using genotypes from Illumina HumanHap370CNV after quality control (Individual Call Rate 97%, SNP Call Rate 98%, MAF 0.01, HWE \( 1 \times 10^{-5} \)); prephasing was performed using ShapeIt v2 and imputation using IMPUTE2 and the ALL (Phase 1 integrated release v3, April 2012) reference panel. Associations with plasma cortisol were analysed in GenABEL.

Detailed Biochemical Studies

More detailed phenotyping was undertaken in samples from 316 participants in the CROATIA-Korcula cohort, comprising 158 age- and sex-matched homozygotes at the top hit variant rs12589136 (53 T/T, 106 G/G; however, one T/T sample had insufficient sample
for CBG measurement resulting in 52 T/T and 106 G/G). An additional 160 randomly selected samples had CBG measured, however two samples failed genotyping resulting in an additional 47 T/G and 111 G/G. Total plasma cortisol was measured with a commercial radioimmunoassay (MP Biomedicals, UK). Total CBG was also measured in CROATIA-Korcula samples using a commercial radioimmunoassay (DiaSource, Louvain-la-Neuve, Belgium). Total cortisol binding capacity was measured using a ligand-saturation assay, with [\textsuperscript{3}H]-cortisol (PerkinElmer Life Sciences, Waltham, MA) as the labelled ligand and dextran-coated charcoal to separate the CBG-bound [\textsuperscript{3}H]cortisol, as previously described\textsuperscript{51}. \(\alpha\)1-antitrypsin was measured with a commercial ELISA (Genway Biotech, San Diego, USA). Unbound free plasma cortisol was calculated from measured total plasma cortisol and total CBG values using Coolens’ equation\textsuperscript{52}. Free cortisol was also measured by ELISA following equilibrium dialysis. Briefly, dialysis tubing (12–14 kD, Medicell, London, UK) was heated to 80°C for 30 min in 2% Na bicarbonate and 1 mM EDTA before overnight dialysis of plasma into phosphate buffered saline containing 1% gelatin at 37°C and measurement of dialysed free cortisol by commercial ELISA (Salimetrics Europe Ltd, Newmarket, UK). CBG were also measured, as previously described\textsuperscript{23}, by ELISAs using monoclonal antibodies 12G2 and 9G12. Antibody 9G12 binds to an epitope in the reactive centre loop (RCL), the elastase cleavage site on CBG, and has been used to infer intact (uncleaved) CBG, whereas antibody 12G2 binds to a distant epitope and measures total (cleaved and uncleaved) CBG.

As CROATIA-Korcula is a population isolate, we used the polygenic and mmscore functions in GenABEL\textsuperscript{50}. All regression equations included the first three principal components and kinship matrix derived from GWAS data in this population and used z-scores of the traits. All variables were normalised using log transformation (cortisol, calculated free cortisol, measured free cortisol, CBG, \(\alpha\)1-antitrypsin), and reported means are geometric means, the ratio of intact/cleaved was normally distributed.
REFERENCES


Chapter 2.4

The low single nucleotide polymorphism heritability of plasma and saliva cortisol levels

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Chapter 2.4

ABSTRACT

Cortisol is an important stress hormone affected by a variety of biological and environmental factors, such as the circadian rhythm, exercise and psychological stress. Cortisol is mostly measured using blood or saliva samples. A number of genetic variants have been found to contribute to cortisol levels with these methods. While the effects of several specific single genetic variants is known, the joint genome-wide contribution to cortisol levels is unclear. Our aim was to estimate the amount of cortisol variance explained by common single nucleotide polymorphisms, i.e. the SNP heritability, using a variety of cortisol measures, cohorts and analysis approaches. We analyzed morning plasma (n=5,705) and saliva levels (n=1,717), as well as diurnal saliva levels (n=1,541), in the Rotterdam Study using genomic restricted maximum likelihood estimation. Additionally, linkage disequilibrium score regression was fitted on the results of genome-wide association studies (GWAS) performed by the CORNET consortium on morning plasma cortisol (n=12,597) and saliva cortisol (n=7,703). No significant SNP heritability was detected for any cortisol measure, sample or analysis approach. Point estimates ranged from 0% to 9%. Morning plasma cortisol in the CORNET cohorts, the sample with the most power, had a 6% [95%CI: 0-13%] SNP heritability. The results consistently suggest a low SNP heritability of these acute and short-term measures of cortisol. The low SNP heritability may reflect the substantial environmental and, in particular, situational component of these cortisol measures. Future GWAS will require very large sample sizes. Alternatively, more long-term cortisol measures such as hair cortisol samples are needed to discover further genetic pathways regulating cortisol concentrations.
INTRODUCTION

Cortisol secretion is regulated by the hypothalamic-pituitary-adrenal axis in response to various biological and environmental factors, including physical stressors such as intensive resistance exercise\(^1\) or injury\(^2\), and psychological stressors such as public speaking and demanding cognitive tasks\(^3\). Cortisol secretion has a marked circadian rhythm: secretion peaks shortly after awakening and then drops throughout the day, reflecting the hormone’s role in regulating energy metabolism\(^4\). Additionally, cortisol is secreted rhythmically resulting in a pulsatile ultradian rhythm\(^5\). The combination of these factors leads to substantial systematic and unsystematic variation of cortisol levels throughout the day.

Cortisol levels can be assessed with a variety of methods, the most common being blood in plasma and saliva samples. Plasma samples represent bound and unbound cortisol concentrations, whereas saliva represents the bioactive free cortisol. These measures have a modest to good correlation\(^6\), \(^7\) and have been associated with various traits and states: BMI\(^8\), cardiovascular risk factors including hyperglycaemia\(^9\), psychiatric disorders, such as post-traumatic stress disorder, schizophrenia or bipolar disorder\(^10\), \(^11\) and treatment response to depression\(^12\). Saliva cortisol can be sampled non-invasively, which may reduce the chance of inducing stress, makes repeated measurements more feasible, and facilitates mapping of day-time profiles. Repeated cortisol measures tend to show higher between-visit reliability than single measures at awakening or 8am\(^13\), \(^14\).

Plasma and saliva cortisol have been investigated in twin studies to determine the extent of the genetic contribution underlying the hormone. For acute plasma cortisol measures, the estimates range from low (14%) to moderate heritability (45%)\(^15\)-\(^17\). Wüst, Federenko, Hellhammer, & Kirschbaum (2000)\(^18\) reported 0% heritability for acute saliva levels at 8am and total day-time profiles, and observed a large contribution of shared environment (>40%). These family studies rely on relatedness information obtained from known familial relationships instead of direct molecular measurements such as SNP arrays. Molecular genetic studies that can clarify the nature and extent of the genetic effects underlying cortisol are lacking, although they could advance our understanding of the genetic contribution to stress vulnerability as assessed by cortisol. A genome-wide association study (GWAS) by the cortisol network consortium (CORNET) successfully detected and replicated one genetic locus associated with morning plasma cortisol levels, suggesting that common autosomal gene variants are associated with this phenotype\(^19\). It is plausible that a substantial number of variants associated with cortisol were not identified due to stringent multiple testing corrections required in GWAS. If this is the case, then the joint effect of all SNPs should be larger than the variance explained by the locus found (<1%).
In the present study, we aimed to quantify the SNP heritability of cortisol, i.e. the variance jointly explained by common autosomal single nucleotide polymorphisms. The SNP heritability information represents a more direct measure of the genetic predisposition to high or low cortisol stemming from additive genetic effects of common gene variants compared to the broad-sense heritability estimated in family studies. SNP heritability can therefore inform future GWA studies about sample size and potential success. We focus on cortisol measured in plasma and saliva measured in elderly participants from the Rotterdam Study and in mixed ages from the CORNET cohorts. This allowed the study of acute morning levels (plasma and saliva) and day-time profiles (saliva) in large sample sizes. SNP heritability can be estimated with different methods. In this study we used genomic restricted maximum likelihood estimation (GREML)\textsuperscript{20} in the Rotterdam Study as well as LD score regression in the CORNET GWAS results.

MATERIALS AND METHODS

Rotterdam Study Participants

The Rotterdam Study is a population-based cohort investigating chronic disease and their risk factors in elderly, see Hofman et al. (2015)\textsuperscript{21} for details. The Rotterdam Study includes 14,926 participants aged 45 and older. Study protocols were approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. Written informed consent was obtained from all participants.

Plasma cortisol information was available in 9836 participants performed in 1997-2008. For 8501, complete information on genetics was available. 2796 participants were removed from GREML analyses due to excessive relatedness (see 2.1.2), resulting in a GREML sample of 5705. In the time adjusted analyses, a further 83 were excluded due to missing information regarding timing of sampling.

Saliva cortisol was available in 2034 participants of which 1982 had complete data on genetics. After removal of 265 participants due to excessive relatedness 1717 individuals remained with acute saliva level upon awakening. Of those, 1541 had also information on later time points for total day-time cortisol computations. See Table 1 for participant characteristics.
Table 1: Descriptive statistics of the Rotterdam Study cortisol measurements and participant characteristics

<table>
<thead>
<tr>
<th>Cortisol Phenotype</th>
<th>Median cortisol levels in nmol/l (25%-75% quantile)</th>
<th>Median age in years (25%-75% quantile)</th>
<th>Sex (% female)</th>
<th>Median time of collection in Hr (25%-75% quantile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>345.6 (281.7;418.1)</td>
<td>63.6 (58.2;72.4)</td>
<td>57%</td>
<td>09:42 (09:00;10:30)</td>
</tr>
<tr>
<td>Saliva (awakening)</td>
<td>13.2 (8.7;18.8)</td>
<td>74.3 (70.5;78.9)</td>
<td>56%</td>
<td>07:30 (07:00;08:06)</td>
</tr>
<tr>
<td>Saliva (AUCg)</td>
<td>7.9 (5.7;10.4)</td>
<td>74.3 (70.5;78.8)</td>
<td>55%</td>
<td>-</td>
</tr>
</tbody>
</table>

**Measurements**

Plasma cortisol was collected from 8:00h to 20:00h. 75% of samples were collected before 10:30 and 99% before 15:30. Cortisol was measured using the LC-MS/MS method with the CHS MSMS Steroids Kit (Perkin Elmer, Turku, Finland) containing $^3$H$_3$-cortisol as internal standard. Chromatographic separation was performed on a Waters (Milford, MA, USA) Acquity UPLC HSS T3 1.8μm column and quantified by tandem mass spectrometry using a Xevo TQ-S system (Waters, Milford, MA).

Sarstedt Cortisol Salivette collection tubes (Sarstedt, Rommelsdorf, Germany) were used to collect saliva after awakening, 30 min after awakening, at 17:00 and at bedtime by the participants. Participants were instructed to note the exact time of saliva collection, and not to eat or brush teeth 15min before collection. An enzyme immunoassay (IBL International Gmbh Hamburg, Hamburg, Germany) was used to analyze the samples. We investigated awakening cortisol levels and diurnal cortisol, calculated by the area under the curve in respect to ground (AUCg).

In the Rotterdam Study genotyping was performed using Illumina HumanHap 550v3 and Illumina HumanHap 610. The genotyped dataset was restricted to persons who reported that they were from European descent. Ethnic outliers were further excluded by removing samples which showed more than 4SD difference to the study population mean on any of the first 4 principal components of a multidimensional scaling analysis. We also excluded samples with gender mismatch and excess autosomal heterozygosity as well as duplicates and monozygotic twins (>97% estimated identity-by-descent proportion). Furthermore, second degree cousins or closer relatives were excluded during the GREML analysis by using a GRM cutoff of 0.025 to avoid bias from shared environment. MACH 1.0 software was used to impute to ~30M SNPs based on the 1000 genomes Phase I version 3 reference panel. SNPs included in imputation met the thresholds minor allele frequency>=1%, Hardy-Weinberg equilibrium p >10E-06, and a SNP call rate >=98.0%.
**GREML**

SNP heritability of the cortisol measurements in the Rotterdam Study were estimated using individual level data with GREML, as implemented in Genome-wide Complex Trait Analysis (GCTA) 1.25.3 (Yang et al., 2011). GREML quantifies how well the similarity in the genotype between study participants explains the similarity in phenotype. Genetic similarity was established by computing a genetic relatedness matrix (GRM). We used 8,131,668 imputed autosomal SNPs to create the GRM, after filtering for imputation quality ($R^2 > 0.5$) and minor allele frequency (MAF) $\geq 0.01$, the GRM was specified as a random effect predicting cortisol levels. To test whether this genetic effect statistically significantly predicts the phenotype, we compared the GRM to a simpler model without the GRM using a likelihood ratio test.

Visual examinations of the total genetic effect and residuals using QQ-plots showed deviations from normality for the saliva measurements. The distribution was normal after square root transformation of hormone levels for saliva cortisol. A constant (+1) was added before transformation to avoid zero values. We report results from analyses on transformed saliva and untransformed plasma levels. Additionally, we performed a power analysis as described by Visscher et al. (2014) 24. The plasma cortisol GREML analyses were well powered to detect 16% heritability (power=80% alpha =0.05 and 2E-5 genetic relationship). The power to detect SNP heritability was less in the saliva GREML analyses and thus these analyses have less precision.

**Covariates and Confounders**

We adjusted the phenotype in all analyses for age, sex and four principal components (PC) of ancestry (computed with GCTA). This was achieved by regressing the phenotype on the covariates and using the residuals as outcome in the GREML analysis. The residuals were computed in R 3.2.3.25 Since plasma cortisol levels were measured in three different Rotterdam Study cohorts, a random intercept on the cohort level was introduced in the regression model of plasma cortisol using the lme4 1.1-10 package 26.

Additionally, we performed a sensitivity analysis with the plasma data aimed at reducing the environmental variance. This model was adjusted for time and fitted in participants with blood sampling before 11am and no self-reported corticosteroid use (n=4,696). To account for non-linear effects, time-of-day was specified using cubic splines with three degrees of freedom. The residuals, representing time-adjusted plasma levels, were then used in further GREML analyses.
**CORNET Consortium Plasma and Saliva Cortisol GWAS**

Detailed description of the CORNET GWAS on plasma cortisol can be found in Bolton et al. (2014)\(^9\). Briefly, basal morning plasma cortisol was measured in 12,597 participants in 11 western European cohorts. Blood samples were collected between 7am and 11am and analyzed using immunoassays. All participants were at least 17 years old and of European ancestry, were not using glucocorticoids, pregnant, or breast feeding. In total 2945 participants (23%) were included from the Rotterdam Study. However, the measurements were collected in a different study wave than the one used for GREML analyses. HapMap-imputed autosomal SNPs were associated with z-scores of log-transformed plasma cortisol levels in an age, sex and time adjusted additive model. The SNP effects were meta-analyzed with a fixed effect model using inverse-variance weighting. After quality control, the data featured 2,660,191 SNPs with minor allele frequency >2%.

In parallel, an additional GWAS of morning saliva levels was performed. This study is unpublished and therefore is presented in more detail. Morning (at awakening) saliva cortisol was measured in 7,703 participants in 8 cohorts: the British 1958 Birth Cohort-Type 1 Diabetes Genetics Consortium (N=1762); the British 1958 Birth Cohort-Wellcome Trust Case-Control Consortium (N=1052)\(^27\); the Netherlands Study of Depression and Anxiety (N=1220)\(^28\); the Netherlands Twin Register (N=162)\(^29\), the Rotterdam Study I (N=1767); the Rotterdam Study III (N=1119); the Multi-Ethnic Study of Atherosclerosis (N=166)\(^30\), and the Tracking Adolescents’ Individual Lives Survey (N=455)\(^31\). Only awakening samples collected before 11 am were included in the analyses. Participants using systemic corticosteroids and pregnant and breast-feeding women were excluded from the analyses. All subjects were at least 16 years old and of European ancestry. Details of the genotyping and imputation are given in Table S2. Genotype quality control was performed in each study separately (HWE P-value >10^-6, MAF >0.01, SNP-call-rate >95%). A z-score was calculated (cortisol at awakening per SD-score in the cohort) to standardize cortisol measurements across cohorts. A linear regression analysis was performed on z-scores of morning saliva cortisol levels adjusted for sex, age and genetic ancestry (cohort specific) using all imputed SNPs.

The meta-analysis was performed with a fixed-effects inverse variance model using the software METAL\(^32\). In addition to study-specific pre-imputation quality control, SNPs with a MAF <0.05 and an observed to expected variance ratio (imputation quality) less than 0.3 were excluded at the meta-analysis level and SNPs. Furthermore, only SNPs with information from 4 or more studies were included, resulting in a final SNP number of 2,156,702 SNPs. Genomic control correction was applied to each study. This GWA morning cortisol saliva meta-analysis has an overlap with the GREML analysis of 1767 participants/measurements (23%) from the Rotterdam Study. QQ and Manhattan plots were created with qqman 0.1.4\(^33\).
LD Score Regression

LD Score regression exploits the relationship between SNP-Phenotype association strengths and linkage disequilibrium (LD) patterns. Some SNPs show stronger associations than expected due to chance. Assuming true causal effects, the SNPs which are in higher linkage disequilibrium (LD) with nearby SNPs are expected to have more inflated test statistics, because they are more likely to tag causal variants with stronger effects. This makes it possible to use a LD score of a SNP, defined as the sum of $r^2$ in a 1cM region, as a predictor of the association strength in a regression. The variance explained by the LD score is equivalent to the SNP heritability estimated by GREML. The advantage of LD score regression is, that it can be conducted with summary data from a GWAS and no individual level information is required. However, this analysis tends to have larger standard errors compared to GREML, which uses individual level data and thus can test SNP heritability effects directly.

The SNP $h^2$ was estimated using LD score regression 1.0.0 in the CORNET GWAS data. Since imputation quality can confound LD score regression results, we restricted the analysis to a list of well-imputed SNPs, as recommended by the software authors. After applying default quality control settings (see Table S3), the final SNP number was 1,028,327 for plasma cortisol and 951,308 for saliva cortisol.

RESULTS

SNP Heritability

Descriptive statistics of the plasma and saliva cortisol levels can be found in Table 1. SNP heritability estimates were low for all cortisol measurement methods, analytical approaches, and cohorts. See Table 2 for full results.

<table>
<thead>
<tr>
<th>Cortisol Phenotype</th>
<th>Analysis Method</th>
<th>Nr. of SNPs</th>
<th>n</th>
<th>SNP $h^2$</th>
<th>SE</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main Analyses:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>GREML</td>
<td>8,131,668</td>
<td>5,705</td>
<td>0.006</td>
<td>0.059</td>
<td>0.460</td>
</tr>
<tr>
<td>Plasma</td>
<td>LD Score</td>
<td>1,028,327</td>
<td>12,597</td>
<td>0.061</td>
<td>0.035</td>
<td>-</td>
</tr>
<tr>
<td>Saliva</td>
<td>GREML</td>
<td>8,131,668</td>
<td>1,717</td>
<td>0.090</td>
<td>0.200</td>
<td>0.329</td>
</tr>
<tr>
<td>Saliva (AUCg)</td>
<td>GREML</td>
<td>8,131,668</td>
<td>1,541</td>
<td>0.041</td>
<td>0.210</td>
<td>0.420</td>
</tr>
<tr>
<td>Saliva</td>
<td>LD Score</td>
<td>951,308</td>
<td>7,703</td>
<td>-0.083</td>
<td>0.060</td>
<td>-</td>
</tr>
<tr>
<td><strong>Sensitivity Analysis:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma-11am</td>
<td>GREML</td>
<td>8,131,668</td>
<td>4,696</td>
<td>0.000</td>
<td>0.073</td>
<td>0.500</td>
</tr>
</tbody>
</table>

Analyses were adjusted for age, sex and ancestry. Plasma cortisol GREML analyses were further adjusted for cohort effects. Additionally, a sensitivity analysis with adjustment for time-of-day and a subset of participants with measurements before 11am and no reported corticosteroid use is reported (Plasma-11am). Negative heritability values can occur for LD score regression analyses due to sampling variance.
**Plasma Cortisol**

We estimated the SNP heritability of plasma cortisol using individual level data of the Rotterdam Study (n=5,705) with GREML. In this cohort approximately 1% [95%CI: 0-12%] of variance in plasma cortisol could be explained by common autosomal gene variants. Adjusting for time of day and excluding participants with plasma cortisol measurements after 11am or those using corticosteroids did not meaningfully change results.

We further investigated the SNP heritability of plasma cortisol in a larger consortium sample: the CORNET cohorts (ncohorts= 11, nparticipants=12,597). We applied LD score regression to estimate SNP heritability of plasma cortisol across multiple cohorts using the summary results of a GWAS meta-analysis. The variance explained for this larger sample was also low with 6% [95%CI: 0-13%].

**Saliva Cortisol**

In addition to plasma cortisol, we estimated the SNP heritability of two saliva cortisol phenotypes: awakening and diurnal levels. First, we estimated the variance explained of saliva awakening levels in the Rotterdam Study with GREML (n=1,717). The heritability in this sample was 9% [95%CI: 0-48%]. Repeating the analysis in the larger CORNET sample (ncohorts= 8, nparticipants=7,703) using LD score regression on GWAS meta-analysis summary statistics showed a negative heritability estimate (-0.0833). Phenotypes with low heritability can be estimated as negative due to sampling variance, which suggests population heritability close to 0 and an upper 95% confidence interval of 3%. Finally, we estimated the SNP heritability of diurnal cortisol levels (AUCg). These were only available in the Rotterdam Study (n=1,541). In this sample the heritability was estimated at 4% [95%CI: 0-45%].

**Morning Plasma and Saliva Cortisol GWAS**

The CORNET GWAS meta-analysis of plasma cortisol, which was previously published (Bolton et al., 2014), identified 4 SNPs in the SERPINA6/SERPINA1 locus, namely rs12589136, rs2749527, rs2749529 and rs11621961.

However, no SNP reached genome-wide significance ($p < 5 \times 10^{-8}$) in the GWAS for awakening saliva cortisol. Table S4 shows results of the top 1000 associated SNPs and Figure 2 displays a Manhattan plot. Two loci showed suggestive associations ($p < 5 \times 10^{-7}$). The T allele of rs1170109 (chr13:42779694) was associated with a 0.12 SD increase in cortisol levels (SE=0.02, $p=3.95 \times 10^{-7}$, MAF=12%, n=7,690) with a homogeneous effect across the cohorts ($I^2=0%$). Several SNPs from the same locus, close to the gene DGKH, showed suggestive effects as well (see Figure 3 for a LocusZoom plot\textsuperscript{35}). The locus was not associated with plasma cortisol ($\beta=0.03$, SE=0.02, $p=0.17$, $I^2=0%$, n=12,592). In the second
locus, the A allele of rs6768297 (chr3:168334386) was associated with 0.34 standard deviations (SD) lower cortisol levels ($SE= 0.06, p= 2.01\times10^{-7}$). Furthermore, the SNP showed a nominally significant ($\alpha =0.05$) association with plasma cortisol in the same direction ($\beta= -0.08, SE= 0.03, p= 0.01, I^2=0\%, n= 11,441$). rs6768297 had a low MAF (6%), high effect heterogeneity ($I^2=85.5\%$) and information was only available in 40% of the sample ($n=3054$). None of the four SNPs associated with plasma cortisol were associated with saliva cortisol (all $p< 0.56$).

The LD score intercept was 1.0031 ($SE= 0.0066$) and 1.0085 ($SE= 0.0073$) for the plasma and saliva GWAS, respectively, suggesting no inflation due to population stratification. The QQ plots also showed no problematic inflation (see Figure 1 for saliva).

![Figure 1](image)

**Figure 1** Quantile-quantile plot of observed $-\log_{10}(p)$ values vs expected $-\log_{10}(p)$ values assuming chance findings. Diagonal line indicates a $p$ value distribution compatible with chance finding. Upward deviations indicate $p$ values more significant than expected.
Figure 2 Manhattan plot of $-\log_{10}(p)$ values vs SNP position. SNPs above the horizontal line indicate suggestive findings ($p < 5 \times 10^{-7}$).

Figure 3 Regional plot around lead SNP rs1170109. $-\log_{10}(p)$ values of rs1170109 and other top1000 SNPs in the region are displayed color coded for strength of correlation.
DISCUSSION

The low heritability of plasma cortisol in two large samples estimated by two different approaches strongly suggests that plasma cortisol is not substantially affected by the additive effects of autosomal SNPs. The same conclusion can be drawn for morning saliva cortisol, which was also estimated by two analytical approaches, and to a lesser extent for diurnal cortisol.

No SNP reached genome-wide significance in a GWAS of morning saliva cortisol levels, which is expected for traits with low SNP heritability analyzed in relatively small samples. Two loci showed suggestive associations. Interestingly, one top SNP rs6768297 lies within the \textit{EGFEM1P} gene, which has a high and specific expression in the pituitary according to RNA expression data (1.5 reads per kilobase per million)\textsuperscript{36,37}. Furthermore, the SNP showed a nominally significant association with plasma cortisol in the same direction as saliva cortisol.

However, the lack of genome-wide significance, low sample size, low MAF and high effect heterogeneity also cast doubt as to whether the rs6768297 association with cortisol would replicate in a completely independent sample. The SERPINA6/SERPINA1 locus identified in the plasma cortisol GWAS\textsuperscript{19} appears to be specific to plasma cortisol levels.

The results are consistent with phenotypic studies indicating that only a small proportion of cortisol variance shows a stable trait-like pattern. In three different studies Ross, Murphy, Adam, Chen, & Miller (2014)\textsuperscript{38} found that 44.4%-75.5% of total day-time cortisol output variance was under day-to-day fluctuations. Studying children through ages 9-15, Shirtcliff et al. (2012)\textsuperscript{39} found that situation-specific environmental influences can explain 52% of cortisol variance (excluding circadian rhythm). The authors conclude that only 13% of the cortisol variance at a given time shows trait-like stability over the years, which coincides with the upper confidence intervals found for the heritability of acute plasma levels. These studies highlight the fact that cortisol secretion and metabolism is a highly dynamic process adapting to not only short-term, but also long-term situational contexts, which results in considerable “noise” in genetic studies.

This notion is supported by the low heritability of the diurnal cortisol measurements. Reducing the within-day variation appears to be insufficient to reduce the contextual noise. This conclusion is further supported by the small effect adjusting for time-of-day had on the plasma cortisol estimates and the low heritability of awakening saliva cortisol. The latter has a precise circadian definition, though sampling can be difficult to time in a home
environment. Furthermore, after excluding participants with plasma cortisol measurements after 11am and corticosteroid use, heritability estimates remained under 1%.

Interestingly, long-term associations between single cortisol measures in adulthood and psychosocial problems and adversities in childhood have been found. The variability might thus reflect environmental exposures, but for genetic studies more long-term profiles of cortisol may be needed. These can be measured using hair samples, which might represent more trait-like effects with less environmental influence. However, long-term environmental contexts spanning months or years also contribute to the cortisol variance and it is unclear yet to what extent 3 to 6 month measurements shall reduce environmental noise.

Therefore there may not be a single simplistic genomic heritability of cortisol levels. It is tempting to speculate that the heritability of other cortisol phenotypes is higher. Indeed the reliability of, for example, the total daily cortisol values (AUCg) is higher than single morning samples, but it represents a distinct feature of the cortisol secretion pattern. The cortisol awakening response or diurnal slopes are two other examples of characterizing diurnal changes. These may show a different balance of genetic and environmental influences than total daily values or hair cortisol. The awakening response or diurnal slopes may show higher heritability than the tested phenotypes, though, it should be noted that they show less stability than total daily output. Another potentially interesting phenotype is cortisol reactivity to various stressors. Here again the heritability may be different and may even change depending on the stressor. Unfortunately, sample sizes for stress reactivity will likely be smaller. Future research is required to determine the SNP heritability of these alternative phenotypes and characterize potential differences between them, although this may be a challenging research field.

The very low diurnal cortisol heritability is in line with a twin-study reporting no genetic effects for day-time profiles. The same study found a non-significant heritability of 26% for awakening cortisol, which is compatible with the non-significant point estimate of 9% SNP heritability in the GREML analysis. Further, the observed 0% to 6% SNP heritability for (mostly morning) plasma and saliva levels (LD score regression) are similar to the 0% and 14% twin heritabilities reported for saliva and plasma morning levels. However, they show a substantial difference to twin studies finding a 45% heritability of acute plasma levels.

SNP heritability is expected to be lower than twin heritability, since this estimate does not include the effects of rare, structural and X-linked variants, which are captured in twin studies. Gene-gene and gene-environment interactions can also substantially increase
standard twin heritability estimates. Alternatively, 45% twin heritability of acute cortisol measurements might be an overestimation, which would be consistent with the fact that the twin studies are highly inconsistent.

The LD score regression and GREML analysis of plasma cortisol in the CORNET and Rotterdam Study samples had good power to detect modest heritability. The negative findings in addition to the convergent evidence from the smaller saliva cortisol samples suggest that acute cortisol measures have low SNP heritability. However, the evidence is less clear for day-time profiles. These were only available in a small sample and have very wide confidence intervals, thus firm conclusions cannot be made. Another limitation is that the CORNET and Rotterdam Study data have an overlap in participants of approximately 20%. The samples were thus not completely independent. However, considering that the majority of the observations did not overlap and the measurements were taken at different times and assessed in different laboratories, the data nevertheless support robustness of the largely negative results.

The findings suggest that common autosomal SNPs are poor predictors of acute cortisol levels. However, predictive power is not equal to importance. Crucial cortisol regulating loci are highly conserved: mammals and fish have a similar stress physiology. Among others, corticotrophin-releasing hormone genes are orthogonal with substantial overlap in amino acid identity. This highlights the importance of cortisol related genes, but also suggests that natural selection restricts the amount of variation and in turn effect sizes and predictive power. This may suggest, that if SNPs are identified despite the low SNP heritability, such as SNPs of the SERPINA6/SERPINA1 locus in the plasma cortisol GWAS, they are all the more important.

Unfortunately, it follows from the presented results that detecting these SNPs will be difficult. Since most SNPs are expected to have a relatively low predictive contribution compared to the environment and stochastic factors, very large sample sizes are probably required to discover further loci. Given the apparent importance of cortisol genetics, GWAS seems nevertheless a worthwhile endeavor to uncover further cortisol related biological pathways.
REFERENCES


Chapter 3

Vascular depression hypothesis epidemiological studies
Chapter 3.1

Silent brain infarcts: A cause of depression in the elderly?

Heidi C. Saavedra Perez, Nese Direk, Albert Hofman, Meike W. Vernooij, Henning Tiemeier, Mohammad Arfan Ikram

ABSTRACT

The present study included 1047 elderly participants. At baseline, brain magnetic resonance imaging (MRI) was performed to detect infarcts and white matter lesions; further, depressive disorders were assessed. Participants were followed up during 3.6 years to determine incident and recurrent depression. We found an increased risk of recurrent depression associated with silent brain infarcts.
INTRODUCTION

A relationship between cerebrovascular disease and depression in the elderly has been established. However, it remains unclear to what extent depression is a direct consequence of vascular damage or a psychological reaction to physical disability caused by stroke.

Studies investigating the relation between cerebral white matter lesions (WMLs) and depression provided evidence that subclinical cerebrovascular disease is associated with depression, although not consistently. Also, silent brain infarcts (SBIs) detected by magnetic resonance imaging (MRI) in the absence of clinical stroke are frequent in depressed patients. The association between SBIs and depression in the elderly has not been studied longitudinally; only the prognosis of prevalent depression in patients with silent brain infarct has been examined. Furthermore, previous studies were based on small, clinical samples. Previous research on the relationship between SBI and the poor prognosis of patients with depression or lack of response to antidepressant treatment prompted us to investigate the possible relationship between SBI and recurrent depression.

Therefore, we aimed to assess whether SBIs and WMLs in community-dwelling elderly increase the risk of incident and recurrent depression.

MATERIALS AND METHODS

Setting

The current study is embedded in the Rotterdam Scan Study in the elderly, a large population-based neuroimaging study.

We randomly selected participants from two large ongoing population-based studies, the Zoetermeer Study and the Rotterdam Study. After 2000, only the Rotterdam Study subcohort was followed. Together with non-participation and death of respondents, this substantial reduction in participants, precluded longer follow-up analysis.

The study was approved by the medical ethics committee of Erasmus Medical Center and participants gave written informed consent.

Participants

The study sample consisted of 1077 non-demented elderly persons aged over 60 years (mean age 70 years, 52% women), who were screened for depressive symptoms and underwent structural MRI to assess cerebral changes at baseline in 1995–1996.
were continuously monitored by reviewing medical records. Follow-up was complete until March, 2000. Participants with no information on depression (n= 16), those who screened positive but without psychiatric diagnostic interview (n= 6), and those diagnosed as having other psychiatric disorders during follow-up (n= 8) were excluded.

**Brain Infarcts and White Matter Lesions**

Axial T1-weighted, T2-weighted, and proton density scans were performed on 1.5 T MRI scanners. Infarcts were defined as focal hyperintensities on T2-weighted images, ≥ 3 mm (Fig 1). A physician scored infarcts, their location and size at baseline. We obtained a history of stroke and transient ischemic attack (TIA) by self-report and medical records. Subsequently, an experienced neurologist categorized the MRI-defined infarcts as silent or symptomatic.

![Fig. 1. Silent brain infarct and white matter lesions in participants of the Rotterdam Study at baseline. Vascular brain changes on 1.5 T MRI are shown. Arrow indicates the abnormality in the image. (A) Periventricular white matter lesions on T2 weighted sequence. (B) Subcortical white matter lesions on T2 weighted sequence. (C) Lacunar infarct on T1 weighted sequence.](image)

We defined SBIs as evidence of one or more infarcts on MRI, without a history of a corresponding stroke or TIA.

WMLs were considered to be present if visible as hyperintense on proton-density and T2-weighted images, without prominent hypointensity on T1-weighted scans. Two raters scored periventricular and subcortical WMLs independently. Severity of periventricular WMLs was rated semi-quantitatively in three regions (grade range 0-9).

**Assessment of Depressive Disorders**

At baseline, we assessed depressive symptoms with a validated Dutch version of the Centre for Epidemiologic Studies Depression (CES-D) scale and by checking indications
of prescribed drugs in all the participants. Prevalent depressive disorders were defined as depressive symptoms (CES-D scores ≥16) or the use of antidepressant medication at baseline.

Information on recurrent and incident depressive disorders during follow-up was established by re-examining 787 participants with the CES-D scale between 1999 and 2000. Screen positives were assessed by a psychiatrist with a Dutch version of the Schedules for Clinical Assessment in Neuropsychiatry (SCAN), to ascertain a Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV) depression diagnosis. In addition, we continuously monitored the general practitioner’s medical records and the Regional Institute for Ambulatory Mental Health records for depressive episodes. Medicals records allow the identification of depressed participants during the interval between assessments.

Incident depressive disorders were defined as depressive disorders during follow-up without depression at baseline. Recurrent depressive disorders were defined as a depressive disorder during follow-up in persons with prevalent depression at baseline. A history of depression was defined as a depressive episode before the baseline examination lasting more than 2 weeks.

**Covariates**

Age, sex, education and cognitive function assessed by Mini-Mental State Examination (MMSE) score were used as possible confounders.

**Data Analysis**

We tested whether the presence of any brain infarcts and the severity of WMLs were associated with incident or recurrent depression with logistic regression. Analyses were adjusted for age, sex, education, and baseline MMSE score.

Separate analyses were run to test the association of symptomatic brain infarcts, SBIs, periventricular and subcortical WMLs and depression. Severe periventricular and subcortical WMLs were defined as ≥5 points for periventricular WMLs and ≥2 ml for subcortical WMLs.

Additionally, in sub-analyses participants with any history of depression were excluded (n= 249). We also repeated the analyses after exclusion of participants who developed dementia during follow-up (n= 34).
RESULTS

At baseline, of the 961 participants without depressive symptoms, 36 persons had symptomatic brain infarcts and 182 had SBIs. Of the 86 participants with depressive symptoms, seven had symptomatic brain infarcts and 25 had SBIs.

During a mean follow-up of 3.6 years, 60 (6.2%) participants without depressive symptoms and 32 (37%) participants with depressive symptoms at baseline were diagnosed with depressive disorders.

Symptomatic brain infarcts and SBIs were not associated with incident depressive disorders in longitudinal analyses. However the presence of SBIs at baseline almost tripled the risk of recurrent depressive disorders (Table 1).

Severe subcortical WMLs doubled the risk of incident depressive disorders whereas severe periventricular WMLs were not related to incident depression. WMLs were not associated with recurrent depression (Table 1).

Exclusion of participants with history of depression and exclusion of participants who developed dementia during follow-up did not change the results (data not shown).

DISCUSSION

We found that older adults with SBIs had an almost three-fold risk of recurrent depression. In contrast SBIs were not associated with incident depression.
So far only an association between SBIs and the prognosis of clinical depression has been studied, showing a poor long-term prognosis and a decreased remission rate in hospitalized patients with SBIs. Our findings are in line with these studies and suggest that SBIs cause depression immediately and lastingly but are not associated with a higher risk of incident depressive episodes during follow-up.

There are several possible explanations for the association between SBIs and recurrent depression in older adults. First, depressive disorders may be a direct consequence of ischemic brain damage. It is known that damage to small vessels supplying subcortical pathways disrupts the neurotransmitter circuitry that is involved in mood regulation. Also, the location and nature of these lesions may affect individuals differently. When the accumulation of the infarcts exceeds a certain threshold, people could become more vulnerable to depression. Second, it has been suggested that depression may contribute to the evolution of vascular risk factors, which in turn increase the risk of cardiovascular disease. Following this reasoning, the underlying vulnerability to depression precipitates the future episode independent of the vascular burden.

Our findings showing the association between severity of subcortical WMLs, which are generally seen as indicating ischemia damage, and incident depression are in accordance with most other studies.

The observed findings remained after exclusion of participants, who developed dementia during follow-up (n= 34), suggesting that associations of SBI and WML with depression are not due to dementia. Moreover, these associations were independent of a history of depression, suggesting that vascular brain damage precedes the depressive disorder, rather than being a consequence of depression.

A potential methodological limitation of our study is the possibility of misclassification. Participants tend to underreport depressive symptoms, and physicians probably underdiagnose depressive disorders, which may have resulted in an underestimation of the true numbers of events. However, in addition to information from medical records, we actively screened participants for the presence of depressive symptoms. Strengths of this study are its large number of elderly people and its prospective population-based design.

In summary, our data show that among elderly persons the presence of SBIs is associated with recurrent depressive disorders.
REFERENCES


Chapter 3.2

Markers of cerebral small vessel disease and severity of depression in the general population

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The vascular depression hypothesis postulates that cerebral small vessel disease can cause or exacerbate depression in elderly persons. Numerous studies explored the association of imaging markers of cerebral small vessel disease including white matter lesions (WMLs) and lacunar infarcts with depressive symptoms or disorders. However, cerebral microbleeds have not been tested in depression. In the current study, we aimed to explore the association of WMLs, lacunar infarcts and cerebral microbleeds with depression continuum in a large population-based sample, the Rotterdam Study. Study population consisted of 3,799 participants (aged 45 or over) free of dementia. WML volumes, lacunar infarcts and cerebral microbleeds were measured with brain magnetic resonance imaging. Depressive symptoms, depressive disorders and co-morbid anxiety disorders were assessed with validated questionnaires and clinical interview. WML volumes and lacunar infarcts were associated with depressive symptoms and disorders. Cerebral microbleeds, especially in deep or infratentorial brain regions, were related to depressive disorders only. Our results indicate that WMLs and lacunar infarcts might be non-specific vascular lesions seen in depressive symptoms and disorders. Association of cerebral microbleeds with more severe forms of depression may indicate impaired brain iron homeostasis or minor episodes of cerebrovascular extraversion, which may play a role in depression etiology.
INTRODUCTION

The co-occurrence of cerebrovascular diseases and depression led researchers to propose the vascular depression hypothesis in late-onset depression. Since the hypothesis was first described by Alexopoulos et al. and Krishnan et al., research on the etiology of vascular depression has been advanced along two conceptually different lines. Some researchers have focused on the localization of the vascular lesions to explain the etiology of the vascular depression. Others have explored the cognitive deficits due to the vascular cerebral lesions predisposing to depression. In the last decade, this approach has led researchers to define “the depression-executive dysfunction syndrome” in which symptoms of executive dysfunctions such as difficulty with planning, organizing, and abstracting are seen as part of clinical depression. Despite the supporting evidence, it is still debatable whether the vascular depression exists as a clinical entity. Extracerebral vascular diseases seem to be less consistently associated with depression in elderly than to cerebral vascular diseases.

While vascular risk factors, lesions and diseases are very common in elderly, the prevalence of depression does not increase in parallel. If the vascular depression hypothesis was of public health importance (i.e., vascular factors strongly predispose, precipitate, and perpetuate depression), the prevalence of depression in elderly would be expected to rise more strongly with age.

Early studies have tested the link between depression and clinically overt vascular events. In these patients, the effects of the functional deficits on depression as a result of the vascular event are difficult to control for. In a recent report of the Rotterdam Study, myocardial infarction was related to depression in men, only when recognized that supporting the importance of psychosocial effects of an overt disease.

Nowadays, a narrower definition of vascular depression hypothesis is used in which the vascular component is considered as clinical or non-clinical cerebrovascular events. Non-clinical vascular events are consisted of imaging findings of cerebral small vessel disease. Such findings occur as a result of hypertension, arteriolosclerosis, inflammation or amyloid deposition in small arteries, arterioles or venules of the brain. These imaging findings are common in the elderly people. The most commonly explored imaging findings of cerebral small vessel disease are white matter lesions (WMLs) and lacunar infarcts. For decades, WMLs have been explored in different severity degrees of depression. Cross-sectional and longitudinal association of WMLs with depressive symptoms, major depressive disorder (MDD), poor treatment response, and occurrence and recurrence of MDD and comorbid anxiety disorders have been demonstrated. Similarly, lacunar infarcts have been related to depressive symptoms, MDD, and recurrence of MDD.
Cerebral microbleeds are now recognized as an imaging phenotype of the cerebral small vessel disease.\(^8,^{20-22}\). Cerebral microbleeds are perivascular collections of hemosiderin induced by prior tiny hemorrhage. Similar to the WMIs and lacunar infarcts, cerebral microbleeds are detected commonly in elderly.\(^{23}\) In general, there are two types of cerebral microbleeds on the basis of location: deep or infratentorial microbleeds and lobar microbleeds. Deep or infratentorial microbleeds were generally related to vascular risk factors whereas lobar microbleeds were associated with cerebral amyloid angiopathy.\(^{24}\) Cerebral microbleeds and their locations have not yet been extensively tested in depressive disorders. Studies assessing the relation between cerebral microbleeds and depressive disorders are mostly limited to the stroke cases in clinical settings.\(^{25-28}\) In a recent longitudinal study, it was shown that cerebral microbleeds were not related to incident depressive symptoms.\(^{29}\) However, the relation of cerebral microbleeds with different severity degrees of depression including depressive symptoms, depressive disorders and comorbid conditions of depressive disorders have not been tested in general population.

The link between cerebral small vessel disease and depressive symptoms has been tested in large population-based studies. However, the association between cerebral small vessel disease and depressive disorders was assessed mostly in small clinical studies.

In the current study, we aimed to test the associations of several imaging phenotypes of cerebral small vessel disease with different severity degrees of depression including depressive symptoms, depressive disorders and depressive disorders with comorbid anxiety disorders in general population. We hypothesized that WMIs, lacunar infarcts, and cerebral microbleeds are all related to the depression continuum.

**MATERIALS AND METHODS**

**Study Sample**

This study was embedded in the Rotterdam Study, a prospective population-based cohort of middle-aged and elderly persons.\(^{30}\) From 2005 to 2008, a random sample within the Rotterdam Study was formed for the regular research center visits. They were invited for brain magnetic resonance imaging (MRI). In total 3,855 participants were involved. Of these, 44 (1.1%) had no depression assessment and 10 (0.3%) persons with dementia were excluded. This left 3,799 people in the study sample.

Of the 3,799 persons in one or more analysis, 3,741 had a valid WML measurement, 3,742 had data on microbleeds and 3,701 participants had data on lacunar infarcts.
The Rotterdam Study has been approved by the institutional review board of the Erasmus University Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports. All participants provided written informed consent after complete description of the Rotterdam Study.

Assessment of Depressive Symptoms and Anxiety Disorders

We diagnosed depressive disorders with a two-step procedure. First, we tested all participants for depressive symptoms using the Center for Epidemiological Studies-Depression (CES-D) scale during the home interview at study entry. A cut-off of 16 was used to define “clinically significant depressive symptoms”. This cut-off score has a very high sensitivity for major depression in older adults in the Netherlands. In the second step, we invited the participants with a CES-D score of 16 or greater to a semi-structured interview, the Schedules for Clinical Assessment in Neuropsychiatry (SCAN). Clinicians were conducted the interviews in close proximity in time to screening. Clinical depressive disorders included DSM-IV-TR–defined major depressive disorder and dysthymia. Thus, both clinically significant depressive symptoms and DSM-IV depressive disorders were assessed in this study.

To determine participants with depressive disorders and comorbid anxiety disorders, we used a slightly adapted version of the Munich version of the Composite International Diagnostic Interview (M-CIDI). The interview was performed by trained interviewers. All DSM-IV anxiety disorders including generalized anxiety disorders, panic disorder with or without agoraphobia, social phobia and specific phobia were assessed except obsessive-compulsive disorder and post-traumatic stress disorder (rare and difficult to diagnose reliably in the general population). We grouped anxiety disorders into a category of “any anxiety disorder”.

Brain MRI

Brain MRI was performed on a 1.5-T scanner (GE Healthcare, Milwaukee, WI) with an 8-channel head coil including T1-weighted, proton-density weighted, fluid-attenuated inversion recovery, and T2*-weighted gradient echo sequences. Post-processing steps have been described elsewhere and include a conventional k-nearest-neighbor brain tissue classifier extended with WML segmentation. Using this classifier, we obtained quantitative measures of WML volume and intracranial volume (in mL).

We defined lacunar infarcts as focal hyperintensities that are \( \geq 3 \) mm on FLAIR and T2-weighted images. An experienced physician scored all infarcts.
All scans were reviewed by 1 of 5 well-trained raters who were blinded to the clinical data. Intra-observer (n=500, 1 rater) and inter-observer (n=300) reliabilities were $\kappa = 0.87$ and $\kappa = 0.85$, which indicates very good agreement. In line with previous studies, we defined categories of microbleeds restricted to a lobar location (strictly lobar microbleeds) and microbleeds in a deep or infratentorial location 37, 38.

**Covariate Assessment**

Age, sex, education, smoking status, hypertension, diabetes mellitus, body mass index, total and HDL cholesterol concentrations and cognitive function were used as potential covariates on the basis of prior literature 39. Education was grouped into eight categories in an ordinary scale from primary education (1) to university level (8) on the basis of the Standard Classification of Education. For the analyses, we further categorized it into three categories; low, intermediate, and high. Smoking status was coded in categories as never, former and current smokers. Systolic and diastolic blood pressures were calculated as the average of two consecutive measurements. Hypertension was defined as a systolic blood pressure $\geq 140$ mm Hg or a diastolic blood pressure $\geq 90$ mm Hg or the use of antihypertensive medication according to pharmacy records. Data on diabetes mellitus was collected on the basis of the general practitioners’ reports and the assessment at the research center. Participants were considered as having prevalent diabetes when they had at least one of the following four criteria: plasma glucose concentration $\geq 7.0$ mmol/L, random plasma glucose concentration $\geq 11.1$ mmol/L, antidiabetic medication, and/or diabetes treatment by diet. Height and body weight were measured. Body mass index was calculated as weight in kilograms divided by height in meters squared. Cholesterol levels were evaluated with an automated enzymatic procedure. Cognitive function was assessed with the Mini-Mental State Examination (MMSE) 40.

**Statistical Analyses**

We log-transformed the WML volumes to achieve a normal distribution. WML volumes were used as continuous measures and lacunar infarcts and cerebral microbleeds were used as binary variables.

To evaluate the cross-sectional associations of the imaging phenotypes of cerebral small vessel disease with depression continuum, we performed a series of analyses. First, we modeled depressive symptoms score continuously (the CES-D score) and tested the associations between the cerebral small vessel disease and depressive symptoms score with linear regression analyses. Second, we used a pre-defined cut-off of 16 for the CES-D to detect participants with clinically significant depressive symptoms and tested the associations of cerebral small vessel disease with clinically significant depressive symptoms using logistic regression analyses. Third, we examined the associations of the cerebral small ves-
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Next, we tested whether any associations of cerebral microbleeds with depression depended on the localization of microbleeds using strictly lobar and infratentorial microbleeds categories.

In the sensitivity analyses, we explored if the associations of cerebral small vessel disease with MDD differed between persons with a comorbid anxiety disorder and those with MDD only; we used the group of persons without depressive symptoms as the reference group for the multinomial logistic regression analyses testing this difference. These sensitivity analyses were adjusted for age and gender. Analysis of WML volume was adjusted for intracranial volume and analysis of cerebral microbleeds was adjusted for intracranial volume and WML volume.

We imputed the missing values on covariates using expectation maximization algorithm. Missing values on covariates were minimal (maximum, 3.1 %). The SPSS software (version 20; SPSS, Chicago, Illinois) was used for statistical analyses.

RESULTS

Characteristics of the study population (n=3,799) are presented in Table 1. Mean age was 58.7 years (standard deviation [SD]= 7.8) and 2070 (54.5 %) participants (n=2070) were women. In total, 322 (8.5 %) participants had clinically significant depressive symptoms, 60 (1.6%) participants had prevalent depressive disorders according to the DSM-IV. Of the 60 participants with major depressive disorder, 31 (51.7 %) had a comorbid anxiety disorder.

A larger WML volume was positively related with the severity of depressive symptoms as indicated by the higher CES-D scores in the fully adjusted model (regression coefficient per ml increase in WML volume= 0.44; 95 % Confidence Interval [CI]= 0.14; 0.75; p= 0.005). Presence of lacunar infarcts was related with high CES-D scores (regression coefficient
for yes versus no = 1.07; 95% CI= 0.04-2.11, \( p = 0.04 \). There was no association of the presence of cerebral microbleed (regression coefficient for yes versus no= 0.17; 95% CI=-0.48 - 0.81; \( p = 0.61 \)) with the severity of depressive symptoms.

Second, we assessed the associations of the cerebrovascular determinants with clinically significant depressive symptoms (CES-D \( \geq 16 \)). Table 2 shows that, consistent with the continuous analyses, WML volume was positively associated with clinically significant

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Study sample N= 3,799</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, mean (SD)</td>
<td>58.7 (7.8)</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>2070 (54.5)</td>
</tr>
<tr>
<td>Education, n (%)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>438 (11.5)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2411 (63.5)</td>
</tr>
<tr>
<td>High</td>
<td>950 (25)</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>1429 (37.6)</td>
</tr>
<tr>
<td>Former smoker</td>
<td>1152 (30.3)</td>
</tr>
<tr>
<td>Never smoked</td>
<td>1218 (32.1)</td>
</tr>
<tr>
<td>Diabetes mellitus (yes) , n (%)</td>
<td>304 (8)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>1834 (48.3)</td>
</tr>
<tr>
<td>Lipid lowering medication, n (%)</td>
<td>831 (21.9)</td>
</tr>
<tr>
<td>Total serum cholesterol, mmol/l, mean (SD)</td>
<td>5.6 (1)</td>
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<tr>
<td>Serum HDL cholesterol, mmol/l, mean (SD)</td>
<td>1.4 (0.4)</td>
</tr>
<tr>
<td>Body mass index (kg/m²), mean (SD)</td>
<td>27.5 (4.2)</td>
</tr>
<tr>
<td>Mini mental state examination score, mean (SD)</td>
<td>28.1 (1.7)</td>
</tr>
<tr>
<td>Depressive symptom score, mean (SD)</td>
<td>5.3 (7)</td>
</tr>
<tr>
<td>Clinically significant depressive symptoms, n (%)</td>
<td>322 (8.5)</td>
</tr>
<tr>
<td>Depressive disorders(^a), n (%)</td>
<td>60 (1.6)</td>
</tr>
<tr>
<td>Cerebral microbleeds (yes), n (%)</td>
<td>538 (14.4)</td>
</tr>
<tr>
<td>Strictly lobar microbleeds, n (%)</td>
<td>371 (9.9)</td>
</tr>
<tr>
<td>Deep or infratentorial microbleeds, n (%)</td>
<td>167 (4.5)</td>
</tr>
<tr>
<td>White matter lesion volume, ml, mean (SD)</td>
<td>4.3 (7)</td>
</tr>
<tr>
<td>Intracranial volume, ml, mean (SD)</td>
<td>1125.7 (123.4)</td>
</tr>
<tr>
<td>Lacunar infarcts (yes), n (%)</td>
<td>184 (4.8)</td>
</tr>
<tr>
<td>Cortical infarct (yes), n (%)</td>
<td>98 (2.6)</td>
</tr>
</tbody>
</table>

\(^a\) Depressive disorders category includes persons with major depressive disorder or dysthymia
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depressive symptoms. Neither lacunar infarcts nor cerebral microbleeds were related with clinically significant depressive symptoms.

Table 2. Associations between the indicators of cerebral small vessel disease and depressive symptoms and clinical depression

<table>
<thead>
<tr>
<th></th>
<th>Clinically significant depressive symptoms a</th>
<th>DSM-IV depressive disorders b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N= 322</td>
<td>N= 60</td>
</tr>
<tr>
<td>White matter lesion volume, ml</td>
<td>3741</td>
<td></td>
</tr>
<tr>
<td>Age and gender adjusted</td>
<td>1.28 1.10-1.48 0.001</td>
<td>1.37 1.18-1.59 &lt;0.001</td>
</tr>
<tr>
<td>Fully adjusted c</td>
<td>1.25 1.08-1.46 0.004</td>
<td>1.40 1.20-1.62 &lt;0.001</td>
</tr>
<tr>
<td>Cerebral microbleeds</td>
<td>3742</td>
<td></td>
</tr>
<tr>
<td>Age and gender adjusted</td>
<td>1.14 0.82-1.58 0.44</td>
<td>1.45 1.02-2.06 0.04</td>
</tr>
<tr>
<td>Fully adjusted c</td>
<td>1.09 0.78-1.53 0.61</td>
<td>1.40 1.01-1.94 0.04</td>
</tr>
<tr>
<td>Lacunar infarcts e</td>
<td>3701</td>
<td></td>
</tr>
<tr>
<td>Age and gender adjusted</td>
<td>1.22 0.71-2.08 0.47</td>
<td>1.78 1.07-2.95 0.03</td>
</tr>
</tbody>
</table>

a Clinically significant depressive symptoms category consisted of participants with a CES-D score ≥ 16. Participants without depressive symptoms were used as a reference group.
b Depressive disorders category consisted of participants with a DSM-IV major depressive disorder or dysthymia. Participants without depressive symptoms were used as a reference group (n=3477).
c Analyses of WML volume were age, sex, education, smoking status, hypertension, diabetes mellitus, BMI, MMSE score and ICV. Analyses of cerebral microbleeds were adjusted for age, sex, education, smoking status, hypertension, diabetes mellitus, BMI, MMSE score and ICV and WML volume.
d Logistic regression analysis was adjusted for age, sex, education, smoking status, hypertension, diabetes mellitus, BMI, and MMSE score.
e Because of the small sample size, age and gender-adjusted model was performed only.

Next, we evaluated the association of the imaging phenotypes of cerebral small vessel disease with DSM-IV depressive disorders. An increase in WML volume was associated with an increased likelihood of DSM-IV depressive disorders (Odds Ratio [OR] per ml= 1.40; 95% CI= 1.20-1.62; p<0.001). Presence of microbleeds increased the likelihood of having DSM-IV depressive disorders 40% in the fully adjusted model (OR= 1.40; 95% CI= 1.01-1.94; p=0.04). Presence of lacunar infarcts was related to depressive disorders (OR= 1.78; 95% CI= 1.07-2.95, p= 0.03).

In the follow-up analyses, we categorized cerebral microbleeds as deep/infratentorial cerebral microbleeds (n=167, 4.5%) or strictly lobar cerebral microbleeds (n=371, 9.9%). We found a borderline association of deep/infratentorial cerebral microbleeds with depressive disorders (OR= 1.41; 95% CI= 1.0-1.99, p= 0.05). There was no association between strictly lobar cerebral microbleeds and depressive disorders even though the effect size
was similar with the results of the deep/infratentorial cerebral microbleeds and depressive disorders (OR= 1.47; 95% CI= 0.69-1.72, \(p\) = 0.72).

**Sensitivity Analyses**

We examined whether markers of cerebral small vessel disease were related to depressive disorders with comorbid anxiety disorders. Presence of cerebral microbleeds was related to increased likelihood of depressive disorders with comorbid anxiety (OR= 2.15; 95% CI= 1.65-2.81, \(p\) < 0.001). WML volume and presence of lacunar infarcts were not related to depressive disorders with comorbid anxiety.

**DISCUSSION**

In this population-based study, the imaging markers of cerebral small vessel disease were differently related to depressive symptoms and disorders. WML volumes were consistently associated to depressive symptoms and MDD. Cerebral microbleeds were related to depressive disorders only, in particular to depressive disorders with comorbid anxiety.

In the last two decades, WMLs have been studied in persons with depression. Investigators repeatedly demonstrated the association of WMLs with clinical and non-clinical presentations of depression. This suggests that WMLs are relatively non-specific vascular pathologies seen in people with depressive symptoms or clinical depression and even in other common psychiatric disorders including anxiety and dementia\(^9, 10, 12, 13, 41-45\). Also, lacunar infarcts were related to depressive symptoms and depressive disorders in the current study. Previous studies mostly explored the association of lacunar infarcts with depressive symptoms. In contrast, studies of DSM-IV depressive disorders are limited. Silent lacunar infarcts, especially when located in the basal ganglia, were related to depressive symptoms in a small clinical study\(^18\). A recent population-based study showed that subcortical infarcts predict incident depressive symptoms\(^29\). Another study of patients with atherosclerosis demonstrated that lacunar infarcts in deep white matter were related with an increase of depressive symptom severity and a more fluctuating course of depressive symptoms during follow-up\(^19\). In the Rotterdam Study, we previously reported that silent brain infarcts predict the recurrence of clinical depression\(^13\).

The underlying mechanism for the relation of WMLs and lacunar infarcts with depression might be the detrimental local effects of cerebral small vessel disease in the frontostriatal and limbic regions. These local damages disrupt the neurotransmitter circuitry involving in
Markers of cerebral small vessel disease and severity of depression in the general population

Mood regulation \(^{46,47}\). This is supported by observation in the current and previous studies that the subcortical WMLs and lacunar infarcts are consistently related to depression \(^{12,13,48}\).

Cerebral microbleeds have not been tested in relation to different severity degrees of depression in a non-clinical sample. Previously, Tang and colleagues \(^{26,27}\) explored cerebral microbleeds in patients with stroke and found that the presence of cerebral microbleeds was related to post-stroke depression. In a longitudinal study of elderly people in general population; van Sloten et al. detected no association between cerebral microbleeds and incident depressive symptoms \(^{29}\). In our population-based study, we observed an association of cerebral microbleeds with depressive disorders in participants free of stroke. Also, there was a relation between cerebral microbleeds and depressive disorder with comorbid anxiety, which indicates a more severe form of depression. This effect was not found when we test the association of cerebral microbleeds with depressive symptoms although continuous analyses generally have more power to detect the associations. This suggests that cerebral microbleeds may be a specific vascular pathology only seen in the most severe form of depression.

Localization of cerebral microbleeds is of importance because they do not only signal an impact on different anatomical regions but this may also reflect different underlying pathological processes of the vascular pathology. Lobar microbleeds are generally related to cerebral amyloid angiopathy whereas deep infratentorial cerebral microbleeds are associated with hypertensive microangiopathy \(^{24}\). We found that deep/infratentorial microbleeds, which are related to cardiovascular risk factors, were associated to depressive disorders. In contrast, lobar microbleeds indicating an amyloid angiopathy were not associated with depressive disorders even though the effect size was similar with the analyses of deep/infratentorial microbleeds. Overall, our findings support a vascular etiology of depression rather than an involvement of the amyloid pathway in the pathology of depression.

These explanations discussed above imply that the direction of mechanisms is from cerebral small vessel disease to depression \(^{13,42,48,49}\). However, it has also been suggested that there is a bi-directional association between small cerebral vascular disease and depression. The mechanisms by which depression may contribute developing cerebral small vessel disease increase are not completely understood, but biological (atrophy in different brain regions, the hypothalamic-pituitary-adrenal axis dysfunction, inflammation) and lifestyle changes (poor health, smoking, less exercise) during depressive episodes are thought to be risk factors to cerebral small vessel disease \(^{23,50-52}\).

The current study has several strengths. First, the study was based on general population, enhancing the external validity of the findings. Second, we evaluated both depressive
symptoms and depressive disorders. Depressive disorders were diagnosed with a clinical psychiatric interview in this population-based study. Additionally, depressive disorder with comorbid anxiety disorder that is considered a more severe form of depression was tested. Evaluating anxiety disorders is not common in population-based studies. Third, large study sample in the analyses of depressive symptoms allowed us to test several covariates. Finally, we were able to evaluate different imaging markers of cerebral small vessel disease, which allowed us to explore different aspects and mechanisms of cerebral small vessel disease such as ischemia and hemorrhage in the same sample.

There are also some limitations that should be considered when interpreting the results. Firstly, this study was not longitudinal. We discussed above that the direction of the temporality is unclear. In the further studies, cerebral microbleeds needs to be tested longitudinally to interpret if these associations are causal. Secondly, number of participants with depressive and anxiety disorders was low, yet we had enough power to detect the association of cerebral small vessel disease and depressive disorders.

In conclusion, our study supports vascular depression hypothesis. Three imaging markers of cerebral small vessel disease in later life were consistently associated to depressive symptoms and depressive disorders. Our study suggests that deep infratentorial microbleeds can index impaired brain iron homeostasis or minor episodes of cerebrovascular extraversion in persons with depressive disorders but more studies are needed to provide evidence on the exact mechanisms and the nature of cerebral small vessel disease in depression.
REFERENCES


Chapter 3.3

Cerebral hemodynamics and incident depression: The Rotterdam Study

Nese Direk, Peter J. Koudstaal, Albert Hofman, Arfan Ikram, Witte J. Hoogendijk, Henning Tiemeier

According to the vascular depression hypothesis, subclinical cerebrovascular disease can cause depression in older adults. To test this hypothesis, several cross-sectional studies have assessed structural brain parameters, but few have examined hemodynamic alterations in the brain.

From the Rotterdam Study, we studied a cohort of 1,494 participants (age 65+) free of depression, dementia, and stroke at baseline. In the middle cerebral artery blood flow velocities and vasomotor reactivity were measured with transcranial Doppler ultrasonography. All participants were repeatedly assessed for depressive symptoms with Centre for Epidemiological Studies-Depression scale (CES-D). Participants with depressive symptoms (CES-D ≥16) had a semi-structured interview, in order to classify the depression according to DSM-IV criteria. All analyses were adjusted for sociodemographics, vascular risk factors, and incident stroke.

Lower peak-systolic, end-diastolic and mean blood flow velocities at baseline were associated with higher Centre for Epidemiological Studies-Depression scale scores at follow-up. Mean blood flow velocity predicted incident depressive symptoms (Odds ratio 0.74, 95% confidence interval 0.60-0.91, \( p = .004 \)) and depressive disorders (Odds ratio 0.83, 95% confidence interval 0.69-0.98, \( p = .032 \)), whereas decreased baseline vasomotor reactivity predicted incident depressive disorders only (Odds ratio 0.66, 95% confidence interval 0.53-0.83, \( p<.001 \)).

Lower blood flow velocity, indicating reduced cerebral metabolism, predicted depressive symptoms and depressive disorders. Reduced vasomotor reactivity, which may indicate cerebral microangiopathy, predicted depressive disorders only, in healthy older adults. These findings provide prospective evidence for vascular depression hypothesis.
INTRODUCTION

The vascular depression hypothesis suggests that subclinical cerebrovascular changes may ‘predispose, precipitate, or perpetuate’ depressive disorders in older adults. This hypothesis is supported by several observations. First, depression is associated with established stroke and more mild manifestations of cerebrovascular diseases such as transient ischemic attacks as well as cerebral infarcts which reflect small-vessel diseases. Second, late-life depression has been associated with white-matter and deep grey-matter lesions and cerebral atrophy without an overt evidence of clinical cerebrovascular disease. Third, an association between peripheral atherosclerosis and depression has been reported repeatedly although, we could not replicate this association in a longitudinal study of the present population using several peripheral atherosclerosis indicators. Finally, studies exploring pathophysiological alterations of the brain showed regional cerebral hemodynamic alterations in individuals with depressive disorders. Cerebral blood flow velocity, which increases during mental activity, and vasomotor reactivity, a compensatory mechanism for maintaining constant cerebral blood flow in cerebral arterioles, are the important cerebral hemodynamic indices. It is known that cerebral hemodynamics are associated with ischemic changes in the brain that may cause stroke or white-matter lesions. Therefore, measuring cerebral hemodynamics allows testing cerebrovascular functions and autoregulation directly before cerebrovascular changes occur, if studies are performed longitudinally. Depressive disorders have been associated with lower global blood flow velocity and vasomotor reactivity in previous cross-sectional clinical studies. However, only depressive symptoms were cross-sectionally associated with reduced blood flow velocity and reduced vasomotor reactivity in a population based study.

Despite the growing evidence, the debate about the causality of such altered cerebrovascular hemodynamics is unresolved. Because studies exploring the association between cerebrovascular hemodynamics and depression were all cross-sectional, it remains unclear whether blood flow velocity and vasomotor reactivity decrease as a result of current depression. Moreover, previous studies were mostly carried out in clinical settings and in small samples, which are more likely to suffer from selection bias. In this study, which was designed to overcome these shortcomings, we postulated that reduced cerebral blood flow velocity and vasomotor reactivity are underlying risk factors for incident depression in healthy older adults.
METHODS AND MATERIALS

Study Setting and Design
This study was part of the Rotterdam Study, a prospective population-based cohort on chronic and disabling diseases in the elderly. Detailed information on the design of the Rotterdam Study has been published elsewhere. The Rotterdam Study has been approved by the institutional review board of the Erasmus University Medical Center and by the review board of the Netherlands Ministry of Health, Welfare and Sports. All participants provided written informed consent after complete description of the Rotterdam Study.

Baseline measurements for the current study were done at the third examination (1997-1999). This included a home interview and a research-centre visit in which cerebral transcranial Doppler ultrasonography was performed and depression screening was introduced. At the fourth examination (2002-2004), depression was assessed during home interviews. Mean duration of follow-up was 4.1 years (standard deviation= 0.5).

Study Population
In total, 4797 participants were involved at the third examination. We attempted to perform transcranial Doppler ultrasonography in 3104 participants. In 998 of these participants (32.2 %), no results were obtained due to window failure on both sides (n= 776), restlessness/discomfort of participants during the procedure (n= 59), participants’ lack of time (n= 5) and other reasons (n= 158). Excluded participants were more likely to be older (p< .001) and female (p< .001). Within 2106 participants with a valid transcranial Doppler ultrasonography measurement, 32 were excluded due to lack of a complete CES-D screening and 2 due to lack of dementia screening at baseline. Further, we excluded screen-positive participants (CES-D score ≥ 16) (n= 116), screen-negative participants (CES-D score < 16) who were on antidepressant treatment (n= 48), participants with dementia (n= 8), and participants with prevalent stroke (n= 43).

Of these 1857 participants, 174 died (9.4%) after the baseline interview and 142 refused to participate (7.6%) to the follow-up interview. At the follow-up visit, 1541 participants were screened with CES-D and 47 did not have a valid CES-D interview. The final study sample thus consisted of 1494 participants. Within this study sample, the following indices of cerebral hemodynamics were available for participants as follows: end-diastolic blood flow velocity, n= 1488; peak-systolic blood flow velocity, n= 1488; mean blood flow velocity, n= 1488; vasomotor reactivity, n= 1445.

Participants with transcranial Doppler ultrasonography measures but who were excluded from the study were older (p< .001) and more likely to be female (p< .001).
Assessment of Depression

Depressive disorders were diagnosed using a two-step procedure. First, all participants were screened for depressive symptoms on the basis of a Centre for Epidemiological Studies-Depression scale (CES-D) during the home interview at the baseline and at the follow-up visit. A cut-off of 16 was used to detect participants with clinically significant depressive symptoms. This score has a very high sensitivity for major depression in older adults in the Netherlands.

In the second step, screen-positive participants were invited for a semi-structured interview, the Schedules for Clinical Assessment in Neuropsychiatry (SCAN), which was performed by trained and experienced clinicians. Depression was categorized according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition, Text Revision (DSM-IV-TR). The category of clinical depressive disorders included DSM-IV-TR defined major depressive disorder, dysthymia, and depressive disorder not otherwise specified (including the former category of minor depression). Participants who were screen-positive according to CES-D, did not meet criteria for diagnoses of major depression, dysthymia, or minor depression, and had at least one core symptom of major depression (i.e. depressed mood or loss of interest), and associated with evidence of dysfunction were categorized as persons with subthreshold depression.

History of depression was evaluated with CES-D and Hospital Anxiety-Depression Scale in the second examination of the Rotterdam Study. A score of 16 or greater for CES-D and 9 or greater for HADS were considered as depressive symptoms.

Transcranial Doppler Assessment

Details on the transcranial Doppler ultrasonography procedure used in the Rotterdam Study have been described earlier. Transcranial Doppler ultrasonography was performed (Multi-Dop X-4, DWL, Sipplingen, Germany) during the third visit of the Rotterdam Study. Cerebral blood flow velocity (cm/sec) was measured in the middle cerebral artery, on both sides when possible. End-diastolic, peak-systolic, and mean cerebral blood flow velocities were recorded automatically. All velocities were measured at a depth of 50 mm or as close as possible. If a flow velocity was measured on both sides, the mean value was used. Otherwise, the available measurement was used.

Cerebrovascular CO₂ reactivity was measured as follows: during the continuous cerebral blood flow velocity measurements, participants first breathed room air through an anesthetic mask that fitted tightly over the nose and mouth. When a steady expiratory end-tidal CO₂ has been obtained, participants inhaled a mixture of 5% CO₂ in 95% O₂ for two minutes. Cerebrovascular CO₂ reactivity was defined as a percentage increase in mean...
cerebral blood flow velocity during inspiration of 5% CO2 divided by the absolute increase in end-tidal CO2 during the same time period (% / kPa).

**Covariate Assessment**

Age, sex, education, cognitive function, incident stroke, hypertension, diabetes mellitus, peripheral arterial disease were considered to be possible confounding variables based on previous publications 5, 22. Education was rated from primary education to university level and then grouped into three categories: low, intermediate, and high education. Cognitive function was evaluated with the Mini Mental State Examination (MMSE). Smoking status was categorized as never, former, and current smoker based on the baseline interview. Participants with no smoking history were categorized as never smokers. Former smokers were categorized in two groups: those quitted smoking at least 10 years ago and less than 10 years ago. Finally, participants who are currently smoking were categorized as current smokers. Both prevalent stroke at baseline and incident stroke at follow-up were obtained through automated linkage of the study database with files from general practitioners, hospital records, and the municipality. Diabetes mellitus was defined as having fasting blood glucose concentrations 11.1 mmol/L or over or the use of antidiabetic medication according to pharmacy records. Blood pressure was measured in the right upper arm with a random-zero sphygmomanometer after the participant had been seated for ≥5 minutes. Systolic and diastolic blood pressures were calculated as the average of two consecutive measurements. Hypertension was defined as a systolic blood pressure ≥140 mm Hg or a diastolic blood pressure ≥90 mm Hg or the use of antihypertensive medication according to pharmacy records. As an indicator of peripheral arterial disease, the ankle-brachial index was used by taking the ratio of the systolic blood pressure measured at the tibial artery to that measured at the right arm. Intima-media thickness was measured as the average of the near and the far wall measurements of both the right and left common carotid artery. B-mode ultrasonography was performed using a 7.5-MHz linear-array transducer (ATL Ultra-Mark IV; Advanced Technology Laboratories, Bethel, Wash) 29.

**Statistical Analyses**

Cerebral hemodynamic variables were used continuously in all analyses. The covariates of age, education, MMSE score, ankle-brachial index and intima-media thickness were also used as continuous variables. Sex, hypertension, diabetes mellitus, smoking status, and new-onset stroke between baseline and follow-up visits were analyzed as categorical variables. We assessed the associations between cerebral hemodynamic measurements and depression in three ways. First, linear regression analyses were performed using CES-D score at follow-up continuously to test the linear association between cerebral hemodynamic variables and depression scores. Second, logistic regression analyses were used to calculate the odds ratios and 95 % confidence intervals for the association between
baseline hemodynamic variables and presence of incident depressive symptoms (CES-D score ≥ 16). The group of screen-negative participants (CES-D score <16) at follow-up was used as a reference category in all analyses. After performing initial unadjusted analyses, all analyses were adjusted for age, sex, education, and MMSE score. Then, additional covariates including ankle-brachial index, hypertension, diabetes mellitus, incident stroke, and smoking were entered into the same model. To examine if intima-media thickness explained any association between cerebral hemodynamics and depressive symptoms, we further adjusted analyses for intima-media thickness. The goodness-of-fit of the fully adjusted models were assessed with the Hosmer and Lemeshow test.

Additional analyses were done to test the effect of previous depressive symptoms on the association between cerebral hemodynamics and incident depression by excluding participants with a history of depression.

Finally, multinomial logistic regression analyses were used to test the association of cerebrovascular hemodynamics with incident clinical depressive disorders and subthreshold depression. Due to the small number of participants and the very limited change of the effect estimate in prior analyses, only age, sex, education level, and MMSE score were used as potential covariates. The goodness-of-fit of the fully adjusted models were assessed with Pearson chi-square test. SPSS version 17 was used for statistical analyses. We applied a Bonferroni adjustment to correct for the multiple testing. Because, mean blood flow velocity was derived from peak-systolic and end-diastolic blood flow velocities, we considered the tests in which these variables were used to predict CES-D scores and incident depressive symptoms (CES-D categorized) as four independent tests ($\alpha/4 = .013$).

**RESULTS**

Table 1 shows the baseline characteristics of the study sample. The mean age was 70.1 years (standard deviation= 5.9) and 47.7% of the participants were female. At follow-up, 124 subjects (8.7%) experienced incident depressive symptoms based on high CES-D score. Among those, 16 individuals refused to participate to the SCAN interview, 36 had no clinical depressive disorder or subthreshold depression, 34 diagnosed with a clinical depressive disorder and 38 had subthreshold depression. Participants without a SCAN interview had higher CES-D scores compared to the participants who completed the SCAN interview (25.6 vs. 22.6), although the difference was not statistically significant ($p= .061$).
First, we studied CES-D scores continuously to test the association of cerebral hemodynamics with depression. Peak-systolic (beta=-0.43 per 1-standard deviation increase, standard error (SE)= 0.18, \( p= .015 \)), end-diastolic (beta=-0.69 per standard deviation increase, SE= 0.18, \( p< .001 \)) and mean blood flow velocities (beta=-0.59 per standard deviation increase, SE= 0.18, \( p= .001 \)) were negatively associated with CES-D scores at follow-up in the unadjusted models. Except the association of peak-systolic blood flow velocity, all of these results remained significant after the Bonferroni adjustment. Peak-systolic (beta=-0.47 per 1-standard deviation increase, standard error (SE)= 0.17, \( p= .007 \)), end-diastolic (beta=-0.47 per standard deviation increase, SE= 0.18, \( p= .011 \)) and mean blood flow velocities (beta=-0.50 per standard deviation increase, SE= 0.18, \( p= .005 \)) were negatively associated with CES-D scores at follow-up in the model which was adjusted for age, sex, level of education, MMSE. In the fully adjusted models, in which ankle-brachial index, hypertension, diabetes mellitus, incident stroke, and smoking were entered into the models, the associations between cerebral blood flow velocities and CES-D score remained significant (beta=-0.54 per 1-standard deviation increase in peak-systolic blood flow, SE=

### Table 1. Baseline characteristics of the study population (1997-1999) (n=1494)

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, (female)</td>
<td>712 (47.7)</td>
</tr>
<tr>
<td>Age (years), mean (SD)</td>
<td>70.1 (5.9)</td>
</tr>
<tr>
<td>Education, n (%)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>336 (22.5)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>956 (64.0)</td>
</tr>
<tr>
<td>High</td>
<td>202 (13.5)</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>424 (28.4)</td>
</tr>
<tr>
<td>Former ≥ 10 years</td>
<td>720 (48.2)</td>
</tr>
<tr>
<td>Former &lt; 10 years</td>
<td>125 (8.4)</td>
</tr>
<tr>
<td>Current</td>
<td>225 (15.0)</td>
</tr>
<tr>
<td>Diabetes Mellitus, n (%)</td>
<td>73 (4.9)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>644 (43.1)</td>
</tr>
<tr>
<td>Ankle-brachial index, mean (SD)</td>
<td>1.05 (0.2)</td>
</tr>
<tr>
<td>Intima-media thickness (mm), mean (SD)</td>
<td>0.86 (0.1)</td>
</tr>
<tr>
<td>Body Mass Index, mean (SD)</td>
<td>26.6 (3.8)</td>
</tr>
<tr>
<td>Mini Mental State Examination score, mean (SD)</td>
<td>28.1 (1.5)</td>
</tr>
<tr>
<td>Mean blood flow velocity (cm/sec), mean (SD)</td>
<td>50.9 (11.3)</td>
</tr>
<tr>
<td>End-diastolic blood flow velocity (cm/sec), mean (SD)</td>
<td>33.0 (8.8)</td>
</tr>
<tr>
<td>Peak-systolic blood flow velocity (cm/sec), mean (SD)</td>
<td>86.8 (18.6)</td>
</tr>
<tr>
<td>Vasomotor reactivity [%/kPa], mean (SD)</td>
<td>4.1 (2.8)</td>
</tr>
</tbody>
</table>
0.17, \( p = .002 \); beta= -0.47 per 1- standard deviation increase in end-diastolic blood flow velocity, SE= 0.19, \( p = .012 \); beta= -0.53 per 1- standard deviation increase in mean blood flow velocity, SE= 0.18, \( p = .003 \). These results were significant after the Bonferroni correction for multiple testing. Baseline vasomotor reactivity was not associated with CES-D scores in any models.

Next, we studied CES-D scores categorically based on the defined cut-off (CES-D \( \geq 16 \)). In unadjusted models, reduced mean blood flow velocity, peak-systolic blood flow velocity, and end-diastolic blood flow velocity were related to incident depressive symptoms. Adjustment for age, sex, level of education, and MMSE score also showed that reduced mean blood flow velocity (odds ratio (OR) 0.74 per 1- standard deviation increase, 95% confidence interval (CI) 0.61-0.91, \( p = .004 \)), peak-systolic blood flow velocity (OR 0.76 per 1- standard deviation increase, 95% CI 0.62-0.92, \( p = .005 \)) and end-diastolic blood flow velocity (OR 0.76 per 1- standard deviation increase, 95% CI 0.62-0.94, \( p = .010 \)) predicted incident depressive symptoms. Adjustment for ankle-brachial index, hypertension, diabetes mellitus, incident stroke, and smoking did not change the results (Table 2). Additional adjustment for IMT also showed that reduced mean blood flow velocity (OR 0.74 per 1-standard deviation increase, 95% CI 0.60-0.91, \( p = .004 \)), peak-systolic blood flow velocity (OR 0.75 per 1-standard deviation increase, 95% CI 0.61-0.91, \( p = .004 \)) and end-diastolic blood flow velocity (OR 0.76 per 1-standard deviation increase, 95% CI 0.61-0.94, \( p = .011 \)) predicted incident depressive symptoms. Results remained significant after the Bonferroni correction for multiple testing. Vasomotor reactivity was not associated with incident depressive symptoms in any models. There was no association between vasomotor

| Table 2. The longitudinal association between cerebral hemodynamic parameters and incident depressive symptoms a |
|---------------------------------|-----------------|------------------|-----------------|------------------|
| Cerebral hemodynamic parameters | n | OR | 95% CI | p  | OR | 95% CI | p  |
| Mean BFV, per SD | 1488 | 0.74 | 0.61-0.90 | .003 | 0.74 | 0.60-0.90 | .003 |
| End-diastolic BFV, per SD | 1488 | 0.73 | 0.60-0.89 | .002 | 0.76 | 0.61-0.94 | .011 |
| Peak-systolic BFV, per SD | 1488 | 0.78 | 0.64-0.95 | .011 | 0.74 | 0.61-0.90 | .003 |
| Vasomotor reactivity, per SD | 1445 | 0.94 | 0.78-1.15 | .549 | 1.03 | 0.85-1.26 | .735 |

a Blood flow velocity (BFV) measurements and vasomotor reactivity were analyzed continuously and divided by 1 standard deviation (SD).

b Adjusted for age, sex, education, diabetes mellitus, hypertension, ankle-brachial index, smoking status, incident stroke, and MMSE score.
reactivity and incident depressive symptoms in the model with additional adjustment for IMT (OR 1.04 per 1-standard deviation increase, 95% CI 0.85-1.26, \( p = .73 \)). The fit of the fully adjusted models were good, as shown by the Hosmer and Lemeshow test (\( p > .050 \)). Excluding participants with a history of depression did not change the results (supplementary table 1).

Next, participants with incident depressive symptoms (CES-D score \( \geq 16 \)) were further classified according to the DSM-IV based depressive disorders. Lower mean blood flow velocity was predictive both for incident depressive disorders and incident subthreshold depression. In these analyses, lower vasomotor reactivity also predicted incident depressive disorders. Vasomotor reactivity was associated with incident subthreshold depression. However, it is notable that this odds ratio is in an unexpected direction as it shows that increased vasomotor reactivity is positively associated with incident subthreshold depression (Table 3). The fit of the models were good as indicated by Pearson chi-square test (\( p > .050 \)).

Table 3. Association of cerebral hemodynamic parameters with subthreshold depression and depressive disorders a,b,c

<table>
<thead>
<tr>
<th>Cerebral hemodynamic parameters d</th>
<th>Subthreshold depression (n=38)</th>
<th>Depressive disorders c (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR 95 % CI</td>
<td>OR 95 % CI</td>
</tr>
<tr>
<td>Mean BFV, per SD</td>
<td>0.77 0.65-0.90 0.002</td>
<td>0.83 0.69-0.98 0.032</td>
</tr>
<tr>
<td>End-diastolic BFV per SD</td>
<td>0.78 0.66-0.93 0.006</td>
<td>0.89 0.74-1.07 0.209</td>
</tr>
<tr>
<td>Peak-systolic BFV, per SD</td>
<td>0.77 0.66-0.91 0.002</td>
<td>0.79 0.67-0.94 0.007</td>
</tr>
<tr>
<td>Vasomotor reactivity, per SD</td>
<td>1.18 1.03-1.34 0.022</td>
<td>0.66 0.53-0.83 &lt;.001</td>
</tr>
</tbody>
</table>

\( a \) All analyses were adjusted for age, sex, education, and MMSE score. 
\( b \) Persons with depressive symptoms according to CES-D were categorized into subthreshold depression and depressive disorders on a psychiatric interview. Persons refused to participate to the interview and persons who did not meet the criteria for subthreshold depression were excluded (n=52). 
\( c \) Major depression (n=15), dysthymic disorder (n=4) and minor depression (n=15) were included in this category. 
\( d \) Blood flow velocity measurements and vasomotor reactivity were analyzed as a continuous variable and divided by 1 standard deviation (SD).

**DISCUSSION**

In this population-based prospective study of older adults, reduced cerebral blood flow velocity predicted incident depressive symptoms, subthreshold depression, and depressive disorders. Lower vasomotor reactivity was not associated with incident depressive symptoms, but with incident depressive disorders only.
Previous cross-sectional findings were supported and extended by exploring the direction of this association in a longitudinal setting. To our knowledge, the current study is the first study supporting a possibly causal association between cerebral hemodynamics and incident depression by a temporal longitudinal design.

In a cross-sectional study, in which all participants were free of common cerebrovascular diseases, reduced vasomotor reactivity was associated with depressive disorder. This was confirmed in another study of the same group examining participants free of vascular risk factors. In a previous publication from the Rotterdam Study we found that persons with depressive symptoms had reduced blood flow velocity and vasomotor reactivity. Overall, these findings from cross-sectional studies of cerebral hemodynamics support the vascular depression hypothesis. But, the temporal direction remained uncertain. Our observations provide empirical evidence that the vulnerability for depression is indeed enhanced by cerebrovascular changes. Moreover, the findings may shed light on the mechanism underlying the cerebrovascular effect on depression. Cerebral blood flow velocity, which is modulated by blood viscosity and vascular tone, reflects cerebral metabolism. Alterations in cerebral blood flow velocity occur as a result of age-related structural and biochemical changes in the cerebral vessels, dysfunction in autonomic neurons, and reduced demand. Insufficient autoregulation of cerebral vessels has been found to be associated with cerebrovascular risk factors, cerebrovascular diseases, and subcortical and periventricular white-matter lesions without an overt disease. Damage to end-arteries, particularly in the frontal-subcortical circuits, may therefore disrupt neurotransmitter systems involved in mood regulation, thus causing depression.

In contrast, vasomotor reactivity, which reflects the compensatory dilatory mechanism of cerebral arterioles to maintain constant cerebral blood flow, may indicate microangiopathy in cerebral vessels. In the current study, reduced vasomotor reactivity predicted depressive disorders only. Participants with depressive symptoms and subthreshold depression are a heterogeneous group, which includes participants with other psychiatric conditions such as prolonged grief, anxiety disorders or participants with stressful life events resulting in depressive complaints. Therefore, we carefully postulate that impaired vasomotor reactivity, which reflects microangiopathy but also reflects autonomous disturbances in cerebral arteries, is a more specific predictor of depressive disorders.

There might be several underlying mechanisms for the observed association between cerebral hemodynamics and incident depression. First, cerebral blood flow velocity has been found to be associated with incident stroke, and depressive disorders are common in patients with stroke. It is possible that altered cerebral blood flow may lead to depression via the development of stroke. However, in the current study, this is unlikely because...
we excluded participants with stroke at baseline and adjusted for any new-onset stroke between baseline and follow-up visits.

The second possibility is that the vascular pathology due to generalized atherosclerosis is the underlying mechanism both for altered cerebrovascular hemodynamics \(^{18}\) and for depression \(^{13,35}\), although peripheral atherosclerosis was not associated with depression in a longitudinal study \(^{14}\). Neither were the results in our study changed by adjustment of ankle-brachial index, a measure of peripheral artery disease, or adjustment for intima-media thickness of common carotid artery, which has been qualified as a marker of cerebral atherosclerosis \(^{36}\). Undetected cerebral atherosclerosis is still a very plausible underlying mechanism \(^{14}\).

The third possible explanation is that the observed association reflects reverse causation, i.e., long-term effects of previous depressive episodes influenced the cerebral hemodynamics at baseline. Indeed the association between depression and cerebrovascular diseases, such as stroke, is bidirectional. Depression increases the risk of stroke and cardiovascular mortality. However, it is still unclear whether subclinical alterations to cerebral hemodynamics seen in depression remain after treatment of depression. Reduced cerebral blood flow velocity and vasomotor reactivity have been found to persist after remission of depression \(^{37}\), although this has also been contested \(^{21}\). In our study, results did not change substantially when participants with a history of depression were excluded. Although, depression in older adults is generally chronic, reversed causality cannot be fully ruled out. on the basis of these additional analyses because depression was assessed approximately 4 years before the baseline. To clarify this point, future studies are needed in which the first-ever depression, further episodes, and remission periods are closely monitored.

Finally, poor health causing hemodynamic alterations and independently related to depression might also explain our findings, although results did not change when vascular risk factors and diseases were controlled for.

The current study has several strengths. First, the large number of participants enabled us to evaluate a large number of covariates including sociodemographic, cardiovascular, cerebrovascular variables. Second, the study was based on general population, enhancing the external validity of the findings. Third, depression was based not only on CES-D scoring, but also on a clinical psychiatric interview. This method let us to evaluate screen-positive participants with depressive disorders. Finally, the prospective design allowed us to assess the causal association between cerebral hemodynamics and depression.
Still, some limitations of the study should also be considered. Due mainly to window failure, which has also been reported in previous studies \cite{38,39}, transcranial Doppler ultrasound measurements were unsuccessful in about one third of the participants. Window failure, which occurred most frequently in older participants and in postmenopausal women, depends primarily on temporal bone thickness \cite{40}. However, it is difficult to imagine how this selection at baseline could have biased the associations observed, although it might influence generalizability of the results. This might have caused true association to be underestimated. Selective drop-out of participants might also have led to the participants with incident depression to be underrepresented, yet we retained enough power to detect the association between vasomotor reactivity and depressive disorders.

In conclusion, the current study supports the vascular depression hypothesis in older adults, and the temporal relation provides support for a causal association between cerebrovascular hemodynamics and depression. Overall, impaired cerebrovascular function without any clinical representation might predispose older adults to depression. Therefore, vascular prevention strategies in older adults might be beneficial and decrease the risk of depression.
REFERENCES


S 1. The longitudinal associations between cerebral hemodynamic parameters and depressive symptom scores and incident depressive symptoms in participants without a history of depression

<table>
<thead>
<tr>
<th>Cerebral hemodynamic parameters</th>
<th>Depression scores Multivariable model</th>
<th>Depressive symptoms (n=90) Multivariable model</th>
<th>n</th>
<th>Beta</th>
<th>Standard error</th>
<th>p</th>
<th>OR</th>
<th>95 % CI</th>
<th>p</th>
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<tbody>
<tr>
<td>Mean blood flow velocity</td>
<td>1270</td>
<td>-0.46</td>
<td>0.18</td>
<td>.012</td>
<td>0.71</td>
<td>0.56-0.91</td>
<td>.006</td>
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<tr>
<td>End-diastolic blood flow velocity</td>
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<td>0.56-0.93</td>
<td>.011</td>
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<tr>
<td>Peak-systolic blood flow velocity</td>
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<td>.025</td>
<td>0.73</td>
<td>0.58-0.92</td>
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<td>Vasomotor reactivity</td>
<td>1232</td>
<td>0.07</td>
<td>0.18</td>
<td>.673</td>
<td>1.02</td>
<td>0.82-1.28</td>
<td>.838</td>
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<td></td>
</tr>
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</table>

*a Blood flow velocity measurements and vasomotor reactivity were analyzed continuously and divided by 1 standard deviation (SD).

*b Adjusted for age, sex, education, diabetes mellitus, hypertension, ankle-brachial index, smoking status, incident stroke, and MMSE score.
Chapter 3.4

Plasma amyloid, depression, and dementia in community-dwelling elderly

Nese Direk, Elisabeth M.C. Schrijvers, Renée F.A.G. de Bruijn, Saira Mirza, Albert Hofman, M. Arfan Ikram, Henning Tiemeier

ABSTRACT

Plasma amyloid beta (Aβ) levels have been associated with an increased risk of Alzheimer’s disease (AD). As depression is common before the onset of AD, a few clinical studies tested the cross-sectional association of Aβ levels with depression in elderly and showed incongruous findings. Hence, we tested the longitudinal association between Aβ levels and depressive symptoms in community-dwelling elderly. The study is embedded in a population-based cohort of 980 participants aged 60 years or older from the Rotterdam Study with Aβ levels. Participants were evaluated for depressive symptoms with the Centre for Epidemiological Studies-Depression scale at baseline and repeatedly over the mean follow-up of 11 years. We first performed cross-sectional analyses. Then, we tested the longitudinal association between Aβ levels and depressive symptoms after excluding participants with dementia during follow-up. In cross-sectional analyses, persons with high Aβ₁-₄₀ levels had more clinically relevant depressive symptoms. However, this association was accounted for by persons with clinically relevant depressive symptoms who developed dementia within the next 11 years. In longitudinal analyses, persons with low levels of Aβ₁-₄₀ and Aβ₁-₄₂ without dementia had a higher risk of clinically relevant depressive symptoms during the follow-up. These findings suggest that the cross-sectional association between high plasma Aβ levels and clinically relevant depressive symptoms in the elderly is due to prodromal dementia. In contrast, the longitudinal association between low plasma Aβ levels and depressive symptoms could not be explained by dementia during follow-up suggesting that Aβ peptides may play a distinct role on depression aetiology.
INTRODUCTION

Two soluble forms of amyloid peptides, amyloid beta 1-40 (Aβ1-40) and amyloid beta 1-42 (Aβ1-42), have been associated with an increased risk of Alzheimer’s disease (AD) \(^1,\) \(^2\). On the basis of the observation that depression is common before the onset of AD, several studies have tested the association of Aβ levels with depression in older adults. The findings of these cross-sectional studies were inconsistent. While high Aβ1-42 levels in people with depression were found in some studies, an association between low Aβ1-42 levels and depression was observed in others \(^3-9\). Moreover, to what extent prodromal dementia contributes to the cross-sectional associations is unknown. A longitudinal study that excluded all participants with dementia at baseline or during 5 years of follow-up showed that higher baseline Aβ1-42 levels were associated with incident depression \(^10\). However, as Aβ levels start to change 5 to 10 years before the onset of AD \(^11\), a longer follow-up is needed to clarify whether this association is independent of the prodromal effects of AD. In addition, it is important to explore Aβ1-40, as it has not been tested longitudinally. We therefore investigated the cross-sectional association between A peptides and clinically relevant depressive symptoms with and without exclusion of persons who developed dementia during the follow-up. Next, we conducted longitudinal analyses excluding those who develop dementia during the follow-up.

MATERIAL AND METHODS

Study Sample

This study is embedded in the Rotterdam Study, an ongoing prospective population-based cohort of older adults \(^12\), which has been approved by the institutional review board of Erasmus University Medical Centre and by the review board of the Netherlands Ministry of Health, Welfare and Sports. All participants provided written informed consent after receiving a complete description of the Rotterdam Study.

Baseline measurements for the current study were performed at the examination round between 1997-1999. The first and the second follow-up examinations were performed between 2002-2004 and 2009-2011, respectively. Mean time interval between baseline and the first interview was 4 years (Standard deviation [SD]= 0.5) and between baseline and the second interview was 11 years (SD= 0.6).

At baseline (1997-1999), 4797 participants were involved in the study. From this source population, we formed a random sample of 1023 people for Aβ1-40 or Aβ1-42 measurements. Twenty-two participants who did not have a valid Centre for Epidemiological
Studies Depression scale (CES-D) score and 9 participants with dementia at baseline were excluded from the current study, leaving 992 participants with at least one measurement of Aβ. Also, we excluded participants with Aβ₁₋₄₀ (n= 12) or Aβ₁₋₄₂ (n= 4) concentrations that were out of the mean range of ± 3 standard deviation (SD). The final population for cross-sectional analyses consisted of 980 participants for whom Aβ₁₋₄₀ levels were available and 970 for whom Aβ₁₋₄₂ levels were available. People who excluded from the baseline random sample (n= 43) were older than the participants (p< .001). There was no significant gender difference between participants and non-participants (p= .881).

For the longitudinal analyses, we further excluded 67 participants with clinically relevant depressive symptoms (CES-D score ≥ 16) at baseline and 107 participants with incident dementia by the end of the second follow-up (2011). Of the remaining 806 participants, 86 died (10.7%) between baseline and the follow-up examinations, 58 refused to participate (7.2%) to any follow-up, and 5 (0.6%) did not have a valid CES-D measurement in any follow-up. This provided an overall sample of 657 participants with a valid depression evaluation and at least one Aβ measure (participants with Aβ₁₋₄₀, n= 657; participants with Aβ₁₋₄₂, n= 650) for the longitudinal analyses of repeated CES-D measure. A flow diagram of the study population at baseline and at follow-up visits was presented in figure 1.

Assessment of Aβ Levels
Fasting morning blood samples were collected in sodium citrate containing vacutainers and put on ice immediately after collection. These samples were centrifuged within 60 minutes, and aliquots of the plasma were stored at -80 °C. A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) method was used to determine Aβ plasma concentrations. The mean coefficients of within- and between-assays variation were 4.4 % and 10.1% for Aβ₁₋₄₀ and 4.9% and 14.8% for Aβ₁₋₄₂. The detection limits were 10–1000 pg/ml for Aβ₁₋₄₀ and 5–100 pg/ml for Aβ₁₋₄₂.

Assessment of Depression
We used the CES-D, a self-report scale, for measuring depressive symptoms at baseline and at follow-up visits. The CES-D interviews were part of the home interviews, which were performed by research assistants who monitored the completion of the questions. The CES-D scores were analysed continuously (depressive symptom scores) and a cut-off of 16 was used to detect participants with clinically relevant depressive symptoms. This score has a very high sensitivity for major depression in older adults in the Netherlands. Incident clinically relevant depressive symptoms were defined in participants free of clinically relevant depressive symptoms at baseline as the CES-D score of 16 or over at one of the follow-up assessments.
For additional analyses, we studied past clinically relevant depressive symptoms. In the previous examination wave of the Rotterdam Study (1993-1995), history of clinically relevant depressive symptoms was evaluated with the CES-D or the Hospital Anxiety-Depression Scale (HADS). We used a cut-off score of 16 for the CES-D and a cut-off score of 9 for the HADS to detect participants with clinically relevant depressive symptoms in the past.

**Assessment of Dementia**

Dementia was assessed with multiple sources including diagnostic evaluation at follow-up visits and continuous monitoring to capture more events. Participants were screened for dementia at baseline and follow-up visits using a 3-step protocol. First, all participants were screened for the cognitive functions using the Mini-Mental State Examination (MMSE) and Geriatric Mental State Schedule (GMS) organic level. Then, participants with a MMSE score < 26 or a GMS organic level > 0 underwent the Cambridge Examination for Mental Disorders of the Elderly. Finally, participants who were suspected to have dementia were examined by an experienced neuropsychologist, if necessary. Additionally, participants were continuously monitored for incident dementia by a computerized system that synchronised the study database with the digitized medical records of general practitioners and the regional Institute for Outpatient Mental Health Care. A consensus panel of a neurologist, neuropsychologist, and research physician decided on the final diagnose on the basis of the DSM-III-R criteria for dementia and the NINCDS-ADRDA for Alzheimer’s disease. Follow-up for incident dementia was complete until January 1, 2011. Participants who were free of dementia at baseline but were diagnosed with dementia during the maximum follow-up of 13 years were considered as participants with prodromal dementia at baseline.

**Assessment of Covariates**

Age, sex, level of education, cognitive functions, creatinine levels, antidepressant use and cardiovascular risk factors were considered as possible confounders. Education was grouped into eight categories in an ordinary scale from primary education (1) to university level (8). The MMSE was used to evaluate cognitive state. Plasma creatinine level, which is considered as a determinant of amyloid beta levels, were measured with an enzymatic in-vitro assay (Roche, Germany). Antidepressant use was determined from the cabinet check at home interviews. Body mass index, smoking status, prevalent stroke, diabetes mellitus and hypertension were considered as vascular risk factors. Height (meters) and weight (kilograms) were measured and body mass index was calculated as weight in kilograms divided by height in meters squared. Smoking status was categorized as never, former, and current smoker, on the basis of the baseline interview. Information about prevalent stroke at baseline was obtained through automated linkage of the study database with files from general practitioners, hospital records, and the municipality. Diabetes mellitus was defined as having fasting blood glucose concentrations ≥ 11.1 mmol/L or the use of antidiabetic...
medication. Hypertension was defined as a systolic blood pressure $\geq 140$ mmHg, a diastolic blood pressure $\geq 90$ mmHg, or the use of antihypertensive medication.

**Statistical Analyses**

$\alpha$ levels were analysed continuously. To approach normality, we log transformed the CES-D scores for the continuous analyses. We also tested the CES-D scores categorically using the established cut-off of 16 for clinically relevant depressive symptoms. $\alpha$ levels had normal distribution when we excluded outliers.

To estimate the cross-sectional associations between $\alpha$ levels and the odds of clinically relevant depressive symptoms, we ran two analyses. First, we included all participants with clinically relevant depressive symptoms as cases at baseline. Second, we defined two groups of cases with clinically relevant depressive symptoms at baseline: 1) persons with clinically relevant depressive symptoms and no dementia during follow-up (n= 54), 2) persons with clinically relevant depressive symptoms and dementia during follow-up (n= 13). For the second analysis, we used multinomial logistic regression analysis to compare the two case groups with the reference group, which consisted of persons free of clinically relevant depressive symptoms and dementia during follow-up (n=806).

We tested the longitudinal associations in those who remained free of dementia during the follow-up. The longitudinal association of $\alpha$ levels with continuously measured depressive symptoms was assessed with the Generalized Estimating Equations (GEE), to combine the scores from follow-up visits. This statistical method increases the power and reduces the type I error caused by multiple testing, but also takes account of the correlation between the repeated depression measures. To study the short- and long-term follow-up associations of $\alpha$ levels with depressive symptom scores at first and second follow-up visits, we additionally performed linear regression analyses. Finally, we used the GEE method with a binary logistic function to predict incident clinically relevant depressive symptoms during the mean follow up of 11 years. Additionally, we used logistic regression analyses to investigate the associations at individual follow-up visits.

All analyses were adjusted for age and gender. Additional covariates including education, MMSE score, plasma creatinine levels, and antidepressant use were entered into the model. To investigate to what extent any association of $\alpha$ levels with depressive symptoms is explained by an effect on vascular risk factors, we additionally controlled the GEE analyses for vascular risk factors including smoking status, body mass index, prevalent stroke, hypertension and diabetes mellitus.
We performed a series of sensitivity analyses. First, to test the possible reverse causality, we excluded participants with a history of clinically relevant depression and performed additional GEE analyses. Second, to test the possible effect of including extreme values of Aβ (those above 3 SD) on the associations, we performed sensitivity analyses assigning the outlier values to the value equivalent to 3 SD and repeated the GEE analyses. Finally, we performed a sensitivity analysis considering antidepressant users at follow-up visits as persons with clinically relevant depressive symptoms. This makes it possible any misclassification of persons with clinically relevant depressive symptoms that were successfully treated.

SPSS version 17 was used for statistical analyses. A two-sided $p<.05$ was considered to indicate statistical significance.

RESULTS

The baseline characteristics of the study sample are presented in table 1. The mean age was 71.6 years (SD= 6.8), and 581 (59.3%) of the participants were female. At baseline, 67 (6.8%) participants had clinically relevant depressive symptoms.
The fully adjusted cross-sectional analyses showed that the likelihood of having clinically relevant depressive symptoms was 34% higher per standard deviation increase in $A\beta_{1-40}$ levels (Table 2). However, when we categorized participants on the basis of whether they developed dementia during follow-up in a further analysis, we found that this association was accounted for by persons with clinically relevant depressive symptoms that preceded incident dementia (prodromal dementia). Per standard deviation increase in $A\beta_{1-40}$ levels were related to an approximately two times higher likelihood of having clinically relevant depressive symptoms in participants who developed dementia during follow-up compared to the non-depressed reference group. $A\beta_{1-42}$ levels were not related with clinically relevant depressive symptoms in the cross-sectional analyses (Table 2).
Table 2. Cross-sectional associations between Aβ levels and clinically relevant depressive symptoms by prodromal dementia status (n=980) a

<table>
<thead>
<tr>
<th></th>
<th>All clinically relevant depressive symptoms b</th>
<th>Clinically relevant depressive symptoms without prodromal dementia c</th>
<th>Clinically relevant depressive symptoms with prodromal dementia c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=67)</td>
<td>(n=54)</td>
<td>(n=13)</td>
</tr>
<tr>
<td>OR 95 % CI p</td>
<td>OR 95 % CI p</td>
<td>OR 95 % CI p</td>
<td>OR 95 % CI p</td>
</tr>
<tr>
<td>Aβ1-40 (per SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>1.20 (0.95 - 1.52) .12</td>
<td>1.07 (0.82 - 1.40) .61</td>
<td>1.65 (1.04 - 2.62) .03</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.34 (1.04 - 1.71) .02</td>
<td>1.17 (0.88 - 1.55) .29</td>
<td>2.22 (1.33 - 3.73) .002</td>
</tr>
<tr>
<td>Aβ1-42 (per SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>1.09 (0.87 - 1.37) .45</td>
<td>0.98 (0.74 - 1.29) .87</td>
<td>1.24 (0.86 - 1.79) .25</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.12 (0.89 - 1.41) .32</td>
<td>1.00 (0.76 - 1.32) .98</td>
<td>1.34 (0.92 - 1.95) .12</td>
</tr>
</tbody>
</table>

Abbreviations: Aβ, Amyloid beta; OR, Odds ratio; CI, Confidence interval; SD, Standard deviation
Model 1 is adjusted for age and gender and model 2 is adjusted for age, gender, education, mini mental state examination score, creatinine levels, and antidepressant use.
Persons with a CES-D score 16 or greater are considered as persons with clinically relevant depressive symptoms.
Logistic regression analyses were used to test the cross-sectional association between Aβ levels and clinically relevant depressive symptoms at baseline in the total study population.
We tested the cross-sectional associations of Aβ levels with clinically relevant depressive symptoms first in persons with and then in persons without prodromal dementia using multinomial logistic regression analyses. We used persons free of clinically relevant depressive symptoms and free of prodromal dementia at baseline as the reference category.

The longitudinal analyses of repeated CES-D measurements showed that high Aβ1-40 and Aβ1-42 levels at baseline were associated with a decreased risk of having high depressive symptom scores. As table 3 shows, lower Aβ1-40 and Aβ1-42 levels were associated with higher depressive symptom scores at the first follow-up in those free of dementia during follow-up visits. At the second follow-up visit, there were also inverse associations between Aβ levels and depressive symptom scores, but neither of these associations was statistically significant. Combining two CES-D measurements in different time points increased the precision of estimate as can be seen by the narrower CIs in GEE analyses when compared to the analyses of the individual follow-up visits.

When we used the CES-D scores categorically, we found consistent results. Lower Aβ1-42 levels were associated with a higher risk of clinically relevant depressive symptoms (Table 3) in the combined analyses. At the first follow-up, there was a borderline significant association between Aβ1-42 levels and incident clinically relevant depressive symptoms, whereas that association was clearly significant at the second follow-up. There was an inverse relation also between Aβ1-40 levels and clinically relevant depressive symptoms at the second follow-up, but this association was not observed when we used the GEE method.
### Table 3. Longitudinal associations between Aβ levels and depressive symptoms in participants free of dementia during follow-up visits

<table>
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<tr>
<th>Depressive symptom scores (continuous)</th>
<th></th>
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<td>Repeated scores a</td>
<td>First follow-up b</td>
<td>Second follow-up b</td>
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<tr>
<td></td>
<td>N</td>
<td>Beta</td>
<td>95 % CI</td>
<td>p</td>
<td>N</td>
<td>Beta</td>
<td>95 % CI</td>
<td>p</td>
<td>N</td>
<td>Beta</td>
<td>95 % CI</td>
<td>p</td>
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<tr>
<td>Aβ1-40 (per SD)</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Model 1</td>
<td>657</td>
<td>-0.09</td>
<td>-0.16; -0.02</td>
<td>.01</td>
<td>638</td>
<td>-0.09</td>
<td>-0.17; -0.02</td>
<td>.02</td>
<td>451</td>
<td>-0.08</td>
<td>-0.17; 0.01</td>
<td>.07</td>
</tr>
<tr>
<td>Model 2</td>
<td>657</td>
<td>-0.10</td>
<td>-0.17; -0.03</td>
<td>.01</td>
<td>638</td>
<td>-0.11</td>
<td>-0.19; -0.03</td>
<td>.01</td>
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<td>-0.08</td>
<td>-0.18; 0.01</td>
<td>.08</td>
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<tr>
<td>Aβ1-42 (per SD)</td>
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<tr>
<td>Model 1</td>
<td>650</td>
<td>-0.10</td>
<td>-0.16; -0.03</td>
<td>.003</td>
<td>632</td>
<td>-0.12</td>
<td>-0.20; -0.04</td>
<td>.004</td>
<td>443</td>
<td>-0.07</td>
<td>-0.16; 0.03</td>
<td>.15</td>
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<tr>
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<td>-0.11</td>
<td>-0.17; -0.04</td>
<td>.001</td>
<td>632</td>
<td>-0.12</td>
<td>-0.20; -0.05</td>
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<td>-0.17; 0.02</td>
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<td></td>
<td>Repeated symptoms a</td>
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<td>Second follow-up b</td>
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<tr>
<td></td>
<td>N</td>
<td>OR</td>
<td>95 % CI</td>
<td>p</td>
<td>N</td>
<td>OR</td>
<td>95 % CI</td>
<td>p</td>
<td>N</td>
<td>OR</td>
<td>95 % CI</td>
<td>p</td>
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<td>Aβ1-40 (per SD)</td>
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<tr>
<td>Model 1</td>
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<td>0.84</td>
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<td>.11</td>
<td>638</td>
<td>0.99</td>
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<td>0.61</td>
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<td>.04</td>
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<td>0.83</td>
<td>0.66; 1.04</td>
<td>.11</td>
<td>638</td>
<td>0.95</td>
<td>0.74; 1.23</td>
<td>.71</td>
<td>451</td>
<td>0.66</td>
<td>0.45; 0.98</td>
<td>.04</td>
</tr>
<tr>
<td>Aβ1-42 (per SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>650</td>
<td>0.69</td>
<td>0.54; 0.88</td>
<td>.002</td>
<td>632</td>
<td>0.77</td>
<td>0.58; 1.03</td>
<td>.09</td>
<td>443</td>
<td>0.57</td>
<td>0.39; 0.84</td>
<td>.01</td>
</tr>
<tr>
<td>Model 2</td>
<td>650</td>
<td>0.67</td>
<td>0.52; 0.86</td>
<td>.002</td>
<td>632</td>
<td>0.73</td>
<td>0.54; 0.99</td>
<td>.04</td>
<td>443</td>
<td>0.60</td>
<td>0.41; 0.89</td>
<td>.01</td>
</tr>
</tbody>
</table>

Abbreviations: SD, Standard deviation; CI, Confidence interval; OR, Odds ratio
Model 1 is adjusted for age and gender and model 2 is adjusted for age, gender, education, mini mental state examination score, creatinine levels, and antidepressant use.

a The generalized estimating equations for repeated CES-D scores or incident clinically relevant depressive symptoms (CES-D ≥ 16) at two follow-up visits were presented. b Linear regression analyses were presented at follow-up visits separately. The CES-D scores were natural log transformed due to the positively skewed distribution. c Logistic regression analyses were presented at follow-up visits separately.
Additional adjustment for vascular risk factors or excluding participants with a history of clinically relevant depressive symptoms did not improve the model in the additional GEE analyses (data not shown). Sensitivity analyses in which we assigned the extreme outlier values equivalent to 3 SD did not change the results of the GEE analyses (data not shown). When we considered antidepressant users as persons with clinically relevant depressive symptoms (cases), our results did not change marginally (data not shown).

**DISCUSSION**

This population-based cohort study explored the cross-sectional and longitudinal associations of Aβ peptides with depressive symptoms in elderly people. Participants with high levels of Aβ₁₋₄₀ were more likely to have prevalent clinically relevant depressive symptoms but only if they developed dementia during follow-up. Longitudinal analyses showed that lower plasma concentrations of Aβ₁₋₄₀ and Aβ₁₋₄₂ were associated with a higher risk of clinically relevant depressive symptoms and lower levels of Aβ₁₋₄₂ predicted incident clinically relevant depressive symptoms during the mean follow-up of 11 years.

The relation of plasma Aβ levels with depression has been tested mostly in cross-sectional studies. A series of studies by one group found lower levels of plasma Aβ₁₋₄₂ in patients with depressive symptoms, whereas plasma Aβ₁₋₄₀ levels were not related to depressive symptoms ⁵⁻⁹. However, these results have not been confirmed by other studies ³, ⁴, ²² and it is not clear whether these associations are due to changes in Aβ levels before the onset of overt dementia. The present cross-sectional analyses showed a positive association between Aβ₁₋₄₀ levels and clinically relevant depressive symptoms. However, this association was accounted for by participants with dementia during follow-up (i.e. those are at the latent or prodromal phase of dementia at baseline) ²³. This finding is also in line with a previous report from the Rotterdam Study, which showed that high Aβ₁₋₄₀ but not Aβ₁₋₄₂ levels predict dementia many years prior to onset of clinical disease ¹.

Plasma Aβ levels change 5 to 10 years before the onset of AD ¹¹, ²⁴. Therefore, it is important to consider the new-onset dementia occurring after baseline when exploring the independent association of Aβ levels with depression. Only one longitudinal study with 5 years of follow-up by Blasko et al. excluded new-onset dementia ¹⁰. Blasko et al. found that high plasma Aβ₁₋₄₂ levels were associated with the first episode of late onset depression, in contrast to our prospective cohort study with a more than 10 years of follow-up for depressive symptoms and dementia.
Several mechanisms may explain the inverse relation between plasma Aβ levels and depressive symptoms that we found in our longitudinal analyses. As cerebral and peripheral levels of Aβ are probably in dynamic equilibrium, any deposition of Aβ peptides in the brain may reduce the plasma levels of Aβ. These brain depositions occur in the neurons and in cerebral vessels which may play a role in the etiology of depression. Neurotoxic effects are characterized by a damage to serotonergic, noradrenergic and cholinergic markers in neurons and increased free radical production. Furthermore, Aβ peptide aggregations in small cerebral vessels can decrease cerebral blood flow and glucose utilization. This may cause white-matter lesions and lacunar infarcts in the brain and precipitate late life depression. However, our analyses showed that adjustment for the common vascular risk factors did not change the results substantially.

Some non-causal explanations should also be discussed. Reversed causality might be responsible for the observed associations. Reversed causality occurs if the outcome is causally related to the exposure. Depressive symptoms before baseline could have altered Aβ levels by the increased platelet activation, which can stimulate Aβ release to the circulation. However, excluding the participants with a history of clinically relevant depressive symptoms did not change the results in our study.

Residual confounding due to unmeasured or unknown confounders e.g. diet, physical activity, non-clinical cerebrovascular changes, etc., which are associated with both Aβ levels and depression, cannot be ruled out and may partially explain our observations.

There are several strengths of the current study. First, the large study sample and existence of numerous data collection enabled us to evaluate a large number of covariates. Second, the population-based setting increased the generalizability of the findings. Third, the prospective design allowed us to assess the temporal association between Aβ peptides and depressive symptoms. Finally, evaluation of the mediating effect of dementia was rendered possible by complete screening for new-onset dementia during follow-up.

Some limitations in our study should be mentioned. Depression was evaluated with the CES-D, which is commonly used in population-based studies to evaluate clinically relevant depressive symptoms. Therefore, it remains unanswered whether these results can be generalized to clinical depressive disorders. Second, loss to follow-up was not very substantial, but may yet be a source of downward bias because participants with clinically relevant depressive symptoms are underrepresented in the study population. However, we had enough power to observe an association between Aβ levels and depressive symptoms. Finally, long time interval between baseline and follow-up interviews may affect our results as those persons with chronic or recurrent depressive symptoms are more likely to be
identified as a case. However, when we consider antidepressant users as persons with clinically relevant depressive symptoms, our results did not change largely.

In conclusion, decreased Aβ levels were associated with clinically relevant depressive symptoms in participants free of dementia during follow-up. Our study provides evidence that Aβ peptides may play a role in aetiology of depression independent of dementia.
REFERENCES


Chapter 4

Depression and genetic epidemiological studies
Chapter 4.1

A combined analysis of two genome-wide association meta-analyses identifies a susceptibility locus for depression continuum

Nese Direk, Stephanie Williams, Jennifer A. Smith, Stephan Ripke, et al.

ABSTRACT

The genetics of depression has been explored in genome-wide association studies that focused on major depressive disorder or depressive symptoms with mostly negative findings. A broad depression phenotype including both phenotypes has not been tested previously using a genome-wide association approach. We aimed to identify genetic polymorphisms significantly associated with a broad phenotype from depressive symptoms to major depressive disorder. We analysed two prior studies of 70,017 participants of European ancestry from general and clinical populations in the discovery stage. We performed a replication meta-analysis of 28,328 participants. SNP-based heritability and genetic correlations were calculated using LD score regression. Discovery and replication analyses were performed using a \( P \)-value based meta-analysis. Lifetime major depressive disorder and depressive symptom scores were used as the outcome measures. The SNP-based heritability of major depressive disorder was 0.21 (SE= 0.02), the SNP-based heritability of depressive symptoms was 0.04 (SE= 0.01), and their genetic correlation was 1.001 (SE= 0.2). We found one genome-wide significant locus related to the broad depression phenotype (rs9825823, chromosome 3: 61,082,153, \( p = 8.2 \times 10^{-9} \)) located in an intron of the \( FHIT \) gene. We replicated this SNP in independent samples \( (p = 0.02) \) and the overall meta-analysis of the discovery and replication cohorts \( (p = 1.0 \times 10^{-9}) \). This large study identified a new locus for depression. Our results support a continuum between depressive symptoms and major depressive disorder. A phenotypically more inclusive approach may help achieve the large sample sizes needed to detect susceptibility loci for depression.
INTRODUCTION

The etiology of depression – a worldwide leading cause of disability \(^1\) – is not well understood. As indicated by family, twin and adoption studies, genetic factors mediate part of vulnerability to major depressive disorder (MDD) with a modest heritability of around 40\% \(^2\). However, we understand little of the specific genetic architecture of MDD. Multiple genome-wide association studies (GWAS) for MDD have been published \(^3\)-\(^{10}\). The largest MDD GWAS was the mega-analysis by the MDD Working Group of the Psychiatric Genomics Consortium (PGC). In that study, more than 9,000 MDD cases and 9,500 control subjects were analyzed, but no association with MDD reached genome-wide significance \(^7\). Recently, the CONVERGE (China, Oxford and VCU Experimental Research on Genetic Epidemiology) consortium identified two genome-wide significant associations in 5,303 Chinese women with severe and recurrent MDD (near the \(SIRT1\) gene, \(p = 2.53 \times 10^{-10}\) and in an intron of the \(LHPP\) gene, \(p = 6.45 \times 10^{-12}\)) \(^{11}\). A GWAS of depressive symptoms (23%-29% heritability) \(^{12,13}\) in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium in approximately 50,000 people from the general population found no genome-wide significant associations \(^{14}\). Owing to the relatively small sample sizes, the previous GWAS of depressive disorders and depressive symptoms were arguably underpowered to detect small genetic effects \(^{15,16}\).

Depression can be conceptualized along a continuum of severity from subthreshold or minor depression to MDD of varying severity (e.g., mild, moderate, severe) \(^{17}\). Using a continuum approach may augment statistical power as sample size can be increased substantially and patients who fall into the ‘grey area’ can be assessed. Several lines of evidence support a depression continuum. In longitudinal studies, there is an increased risk of MDD in patients with minor depression and subthreshold depression \(^{18,19}\). Statistical studies of disorder classification (taxometric) suggested that severity of depression is continuously distributed and there is no discontinuity in the latent structure of depression \(^{19,20}\). Family studies report that relatives of probands with milder forms of depression have greater risk of MDD compared to relatives of probands without any mood disorders \(^{21-24}\). A higher number of depressive symptoms is related to greater disability, worse quality of life, and a higher mortality risk \(^{18,25-29}\). MDD and continuous measures of depression are highly correlated, and severity of depressive symptoms along the continuum is linear \(^{30,31}\).

The goal of the current study was to combine the results of the largest GWAS using categorical lifetime MDD and continuous measures of depression to identify genetic variants underlying the entire depression continuum.
METHODS AND MATERIALS

Study Design and Samples
This study was a collaboration between investigators on the PGC MDD and CHARGE genome-wide association meta-analyses (GWAMA). In the discovery phase, we aggregated two GWAMA published in 2013. Basic descriptive features and phenotype definitions of the contributing samples are provided in Supplemental Table S1. The mega-analysis of MDD consisted of nine studies of 9,240 cases meeting international criteria for lifetime MDD and 9,519 healthy control subjects. The CHARGE meta-analysis of depressive symptoms included 22 cohorts and comprised 51,258 persons. Each cohort contributing to the GWAMA of the PGC and CHARGE were distinct. In the replication analyses, 16 case-control studies with DSM-IV MDD (6,718 cases and 13,453 controls) were included along with 8,157 subjects from the general population with assessment of depressive symptoms. All subjects were of European ancestry. Institutional review boards approved all studies, and all participants provided written informed consent.

Phenotype Characteristics
In the PGC GWAMA, MDD was established with structured clinical interviews (e.g., Clinical Interview Schedule-Revised, Diagnostic Interview for Genetic Studies, and the Structured Clinical Interview for DSM-IV). All clinical evaluations were made by experienced clinicians/interviewers. Most cases were ascertained from clinical sources. Controls were screened in most of the studies to require the absence of MDD and recruited from the general population. Full details about the PGC samples can be found in the previous publication. In the CHARGE GWAMA, depressive symptoms were assessed with validated questionnaires. Measures include the Center for Epidemiological Studies-Depression scale, Geriatric Depression Scale, Patient Health Questionnaire-9, and the Beck Depression Inventory-II, mostly assessing depressive symptoms during previous weeks rather than lifetime MDD. Persons with schizophrenia, bipolar disorder, and dementia were excluded. Persons aged 40 years or older and with genotype data and depressive symptom score were included.

The 16 MDD case-control replication samples were part of an expanded but unpublished PGC MDD analysis. MDD was diagnosed with interviews. In the depressive symptom replication cohort, the Health and Retirement Study, the 8-item Center for Epidemiological Studies-Depression scale was applied. Respondents were excluded if they were less than 40 years of age or evidence of cognitive impairment.

Genotyping and Imputation
In the PGC samples, (Supplemental Table S1), individual genotypes were assembled, processed through a central quality control pipeline and imputed using the CEU (Central
Europe) and TSI (Toscani in Italy) HapMap3 reference panels. Quality control procedures were extensive. In the CHARGE cohorts, genotype quality control and imputation were conducted in each study separately. The imputation reference was the HapMap2 CEU panel. In the MDD replication cohorts (Supplemental Table S3), imputation was performed using IMPUTE2 or SHAPEIT (chunk size of 3 Mb and default parameters). The imputation reference set consisted of 2,186 phased haplotypes from the 1000 Genomes Project. In the Health and Retirement Study, imputation was performed using the HapMap2 CEU reference panel.

**Statistical Analyses**

Linkage disequilibrium score regression was used to compute the single nucleotide polymorphism (SNP)-based heritability and the genetic correlation using the 1000 Genomes CEU reference panel.

In the PGC GWAMA, a logistic regression analysis was used to test the association between MDD and imputed SNP dosages under an additive model and adjusting for study indicators and five principal components. In the CHARGE GWAMA, a linear regression analysis was applied to test the association of depressive symptom score on imputed SNP dosages in the contributing studies adjusting for age and sex. Analyses were adjusted for principal components for most, but not all, cohorts in the CHARGE GWAMA. A p-value-based meta-analysis was applied in the CHARGE GWAMA. Effect size estimates were based on a dichotomous outcome in the PGC and on a continuous outcome in the CHARGE GWAMA. To combine these effect estimates, a p-value-based meta-analysis weighted by sample size with METAL was used. This method allows different weights for each study and takes into account the direction of effect at each SNP. To specify the direction of the effect, the PGC used the logistic regression coefficient beta and the CHARGE used Z-scores. Weights were based on the number of the MDD cases in the PGC study (n= 9,240), and the number of individuals in the CHARGE with clinically significant depressive symptoms (n= 5,976) using population-specific cutoff scores of the questionnaires was considered for weighting. To test whether the results are affected by different sample size weightings, equal weights per study, or no weight as suggested by Stouffer et al., we carried out a series of sensitivity analyses.

We selected the genome-wide significant SNPs in two loci from the discovery stage for replication. After analyzing these data, we performed a p-value-based meta-analysis combining all replication samples. Further, we analyzed the results of the discovery and all replication samples weighting for number of cases.
RESULTS

In the discovery stage, we performed a GWAMA in 70,017 participants of European ancestry by combining the PGC MDD \(^7\) and CHARGE GWAMA \(^{14}\). We applied a linkage disequilibrium score regression to the summary statistics from each study to compute the SNP-based heritabilities and the genetic correlation. As reported previously \(^{35}\), the SNP-based liability scale heritability of MDD was 0.2 (SE= 0.02) for 20% of prevalence. The lambda was 1.1 and the regression intercept was 1.0 (SE= 0.01). The SNP-based heritability of depressive symptoms was 0.04 (SE= 0.01). The lambda was 1.1 and the regression intercept was 1.0 (SE= 0.01). The SNP-based heritability of the broad depression phenotype was 0.3 (SE= 0.04). MDD and depressive symptoms showed significant co-heritability (1.001, SE= 0.2, z-score= 4.6, \(p=4.6\times10^{-6}\)). This result supports the contention of a continuum between depressive symptoms and MDD. However, the genetic correlation should be interpreted carefully because linkage disequilibrium score regression is quite sensitive to environmental confounding, and like twin studies, often lacks precision. In addition, different evaluation methods of the depression phenotypes might cause different genetic correlation estimates that cannot easily be compared.

We conducted a meta-analysis of the PGC MDD and the CHARGE depressive symptoms GWAMA using a weighted, \(p\)-value-based meta-analysis. The results are summarized in Figure 1 and Supplemental Figures S1 to S3. The combined meta-analysis was conducted for 918,921 SNPs. Two loci were genome-wide significant: an SNP in an intron of the \(F\)HIT gene (rs9825823, chromosome 3: 61,082,153, \(p=8.2\times10^{-9}\)) and an SNP in an intron of \(P\)LEK2 (rs9323497, chromosome 14: 67,873,128, \(p=3.3\times10^{-8}\)) (Table 1). All SNPs with a \(p\) value of association <5x10\(^{-5}\) are presented in Supplemental Table S2. Using different weights or Stouffer’s unweighted method had only slight effects on the results (data not shown). Supplemental Figures S4 and S5 show forest plots for two SNPs shown in Table 1.

Table 2 presents the replication analyses and the meta-analysis of discovery and replication results. One of the genome-wide significant variants within the \(F\)HIT gene (rs9825823) was associated with depression continuum in the replication cohorts (z-score=2.4, \(p=0.02\)). The result of the final meta-analysis of discovery and replication samples also indicated a positive replication as indexed by a lower \(p\) value (z-score=6.1, \(p=1.0\times10^{-6}\)). This SNP had a positive association with depressive symptoms in the CHARGE study (\(p=5.5\times10^{-4}\)), and a similar pattern was observed in the PGC study (\(p=4.1\times10^{-6}\)). The SNP in an intron of \(P\)LEK2 (rs9323497) was not related to depression continuum significantly (z-score= 0.2, \(p=0.9\)).
A genome-wide association study of depression continuum

Figure 1: Manhattan Plot. The x-axis represents the chromosomal position for each SNP, and the y-axis represents the $-\log_{10}(p)$ value for association with depression.

Table 1. Meta-analysis results of the PGC MDD GWAMA and the CHARGE depressive symptoms GWAMA(discovery)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chr</th>
<th>BP</th>
<th>Closest Gene</th>
<th>Location</th>
<th>Allele</th>
<th>MAF</th>
<th>Direction</th>
<th>$z$-score</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9825823</td>
<td>3</td>
<td>61,082,153</td>
<td>FHIT</td>
<td>intron</td>
<td>T/C</td>
<td>0.46</td>
<td>++</td>
<td>5.8</td>
<td>8.2x10^{-9}</td>
</tr>
<tr>
<td>rs9323497</td>
<td>14</td>
<td>67,873,128</td>
<td>PLEK2</td>
<td>intron</td>
<td>T/C</td>
<td>0.05</td>
<td>++</td>
<td>5.5</td>
<td>3.3x10^{-8}</td>
</tr>
</tbody>
</table>

Chr: chromosome, BP: base pair, MAF: minor allele frequency (GRCh37/hg19). Allele: Minor/Major on the + strand. Direction of effect: CHARGE (N=51,258; continuous outcome analysis), PGC (n=18,759; of which 9,240 were MDD cases). * This analysis was weighted for number of participants with clinically significant depressive symptoms in the CHARGE study (n=5,976) and number of cases in the PGC study (n=9,240).

We performed an additional replication analysis of our two genome-wide significant SNPs using the publicly available data of the recently published GWAMA of depressive disorders in a sample of Chinese women (the CONVERGE study) 11. In CONVERGE, rs9825823 (odds ratio= 1.01, $p= 0.12$) and rs9323497 (odds ratio= 0.97, $p= 0.0002$, with a different direction of association than in our discovery sample) were not related to depression at the genome-wide significance level, although the latter reached nominal significance. However, in the joint meta-analysis of the Health and Retirement Study, PGC MDD study and the CONVERGE study, we found that the association between rs9825823 and the
### Table 2. Replication analyses and final meta-analysis of discovery and replication stages

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chr:BP</th>
<th>Allele</th>
<th>Direct.</th>
<th>p</th>
<th>Direct.</th>
<th>p</th>
<th>Direct.</th>
<th>p</th>
<th>MAF</th>
<th>Direct.</th>
<th>z-score</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9825823</td>
<td>3:61,082,153</td>
<td>T/C</td>
<td>+++-----+++-----+++</td>
<td>0.04</td>
<td>+</td>
<td>0.2</td>
<td>++</td>
<td>0.02</td>
<td>0.46</td>
<td>+++</td>
<td>6.10</td>
<td>1.0x10^-9</td>
</tr>
<tr>
<td>rs9323497</td>
<td>14:67,873,128</td>
<td>T/C</td>
<td>++++++----+++-----++</td>
<td>0.8</td>
<td>-</td>
<td>0.8</td>
<td>+</td>
<td>0.9</td>
<td>0.05</td>
<td>-++</td>
<td>4.61</td>
<td>4.0x10^-6</td>
</tr>
</tbody>
</table>

Chr: Chromosome, BP: base pair, MAF: minor allele frequency (GRCh37/hg19), Direct=Direction. Allele: Minor/Major on the + strand.  


- Order of the studies: PGC-replication Health and Retirement Study.  

- Order of the studies: PGC-replication, Health and Retirement Study, combined meta-analysis of discovery samples.
A genome-wide association study of depression continuum (z-score= 2.85, p= 0.004) was slightly stronger than our initial replication analysis. When these replication and discovery samples were combined, the association with our top hit also became stronger (analyses without the CONVERGE data: z-score= 6.1, p= 1x10⁻⁹; with the CONVERGE data: z-score= 6.2, p= 6.8x10⁻¹⁰). Results of additional replication analyses are given in Supplemental Table S4.

**DISCUSSION**

We report the results of a combined GWAMA of depression continuum including MDD (18,759 cases and control subjects) and depressive symptoms (51,258 participants). In the discovery stage, we found genome-wide significant associations in the *FHIT* and *PLEK2* genes. One SNP in the intron of the *FHIT* gene showed a significant association in the combined analysis of discovery and replication samples of MDD and depressive symptoms samples, and exceeded a genome-wide significance threshold.

The significant locus (rs9825823, chromosome 3: 61,082,153) maps to the intronic region of the *FHIT* gene, a tumor suppressor protein implicated in several cancers. *FHIT* is expressed in multiple brain regions (amygdala, anterior cingulate cortex, caudate nucleus, prefrontal cortex, hippocampus, and hypothalamus, http://www.gtexportal.org/home/gene/FHIT, accessed 10.07.2016). It plays an important role in oxidative stress and level of DNA damage, biological processes implicated in MDD. *FHIT* is a circadian clock modifier gene and has been related to daytime sleepiness, which may be salient to the etiology of depression.

In a GWAS of recurrent, early-onset MDD, three SNPs located in the *FHIT* gene were among the strongest associations in the overall and sex-stratified analyses although none had genome-wide significance. Genetic variants located in *FHIT* have been reported in genetic studies of anxiety, autism, mental stress, comorbid depressive syndromes and alcohol dependence, citalopram-induced side effects and in a latent class analysis of MDD symptoms, but none has met genome-wide significance.

Several methodological aspects should be discussed. First, we evaluated depression continuum by combining cases from clinical populations diagnosed with MDD and participants from the general population who had been assessed for depressive symptoms. Such an inclusive approach may increase heterogeneity of the phenotype especially because lifetime MDD was evaluated, whereas depressive symptoms indicate past weeks only. If anything, such approach would cause an underestimation of the effects because less information on depressive symptoms was obtained. However, the advantages of a large
sample can outweigh the disadvantages of a less precisely defined phenotype. This has been observed in the GWAS of educational attainment that was successfully used as a proxy for intelligence\textsuperscript{47}. Our additional replication analysis showed that increasing the sample size yielded a stronger association of the top hit with depression continuum. It is complex to calculate statistical power of the current analysis because quantitative and qualitative measures were combined. In the current study, a genetic association with the depression continuum may reflect an effect on broad depressive phenotypes but could also be accounted for by an association with low levels of general well-being (12-18\% heritability) that co-occur with depressive symptoms\textsuperscript{48}. Second, we used a $p$-value-based meta-analysis, because effect estimates could not be directly evaluated in a straightforward manner. Third, the heterogeneity of the imputation methods used in the PGC and CHARGE discovery samples might reduce the statistical power. However, different imputation references did not change the results in the published PGC MDD study\textsuperscript{7}.

In conclusion, in this large GWAMA of a broad depression phenotype, we detected a locus associated with depression in clinical and general population samples. Our results suggest the importance of a broader depression phenotype to identify genetic variants underlying depression. Large samples with different depression phenotypes may also help disentangle the genetic background of different forms of depression.
REFERENCES


Chapter 4.2

Somatic, positive and negative domains of the Center for Epidemiological Studies Depression (CES-D) scale: A meta-analysis of genome-wide association studies


* Contributed equally.

ABSTRACT

Major depressive disorder (MDD) is moderately heritable, however genome-wide association studies (GWAS) for MDD, as well as for related continuous outcomes, have not shown consistent results. Attempts to elucidate the genetic basis of MDD may be hindered by heterogeneity in diagnosis. The Center for Epidemiological Studies Depression (CES-D) scale provides a widely used tool for measuring depressive symptoms clustered in four different domains which can be combined together into a total score but also can be analysed as separate symptom domains. We performed a meta-analysis of GWAS of the CES-D symptom clusters. We recruited 12 cohorts with the 20- or 10-item CES-D (32,528 persons). One single nucleotide polymorphism (SNP), rs713224, located near the brain-expressed melatonin receptor (MTNR1A) gene, was associated with the somatic complaints domain of depression symptoms, with borderline genome-wide significance ($p_{\text{discovery}} = 3.82 \times 10^{-8}$). The SNP was analyzed in an additional five cohorts comprising the replication sample (6,813 persons). However, the association was not consistent among the replication sample ($p_{\text{discovery+replication}} = 1.10 \times 10^{-6}$) with evidence of heterogeneity. Despite the effort to harmonize the phenotypes across cohorts and participants, our study is still underpowered to detect consistent association for depression, even by means of symptom classification. On the contrary, the SNP-based heritability and co-heritability estimation results suggest that a very minor part of the variation could be captured by GWAS, explaining the reason of sparse findings.
INTRODUCTION

Genetic factors play an important role in the susceptibility to depression. A meta-analysis of twin studies on major depressive disorder (MDD) estimated a heritability between 31 and 42%. The success of genome-wide association studies (GWAS) aiming to find genes underlying vulnerability for depression, however, has been limited; the most promising findings to date are poorly replicated and explain only a small portion of this heritability. This may be explained by the polygenic architecture of the trait as well as difficulties in diagnosis. A validated biomarker for depression does not exist and the diagnosis is based solely on symptoms. Such symptoms include depressed mood states, loss of interest in activities, feelings of worthlessness or inappropriate guilt, recurrent thoughts of death, poor concentration, and somatic symptoms such as changes in appetite, sleep patterns, fatigue, and weight gain or loss. Depression can manifest with different patterns of symptoms, and such phenotypic heterogeneity may reflect genetic heterogeneity. It is plausible that different genetic pathways are associated with the various symptom clusters, and analyses of more narrowly defined phenotypes may reduce genetic heterogeneity. Indeed, the diverse domains of complaints, which result in variations in presentation of the disease within and between populations, may lead to problems for gene discovery. A focus on outcomes based on depressive symptoms and endophenotypes has been shown to increase power in association studies. However, the genetic architecture of these outcomes is also complex and may involve the effects of multiple common variants.

Depressive symptoms can be measured by questionnaires, such as the Centre for Epidemiological Studies Depression (CES-D) scale, which shows moderate heritability. The CES-D scale measures symptoms clustered in somatic complaints, lack of positive affect, negative affect and interpersonal problems domains, which are usually combined into a single score. The CES-D subscales can also be analyzed separately in order to focus on the specific symptom domains. We conducted a meta-analysis of GWAS of specific symptom domains measured by CES-D scale. The discovery set consisted of 12 cohorts (n= 32,528) and the replication set consisted of five cohorts (n= 6,813).

MATERIALS AND METHODS

Table 1 summarizes the characteristics of the discovery and replication cohorts from the Cohorts for Hearth and Aging Research in Genetic Epidemiology (CHARGE) Consortium. The main aim of CHARGE is to facilitate GWAS meta-analyses and replication opportunities among multiple large and well-phenotyped longitudinal cohort studies. The discovery sample consisted of the CHARGE cohorts with eligible 20-item CES-D (CES-D-20) data.
These cohorts were the Baltimore Longitudinal Study of Aging (BLSA)\textsuperscript{13}, the Dortmund Health Study\textsuperscript{14, 15}, the Erasmus Rucphen Family Study (ERF)\textsuperscript{16, 17}, the National Heart, Lung, and Blood Institute’s Framingham Heart Study (FHS)\textsuperscript{18-20}, the Helsinki Birth Cohort Study (HBCS)\textsuperscript{21}, European ancestry participants from the Health, Aging and Body Composition study (HEALTH ABC-Eur)\textsuperscript{22}, the Rotterdam Study I-II and III (RS I-II and RS-III)\textsuperscript{23} and SardiNIA\textsuperscript{24} and two studies in which the symptoms of depression were measured with the 10-item version of CES-D (CES-D-10): the Atherosclerosis Risk In Communities (ARIC) study\textsuperscript{25} and the Swedish Twin Registry (STR) study\textsuperscript{26}. FINRISK\textsuperscript{27}, the Health and Retirement Study (HRS)\textsuperscript{28, 29}, Invecchiarre in Chianti (InCHIANTI), and the Memory and Aging Project and Religious Order Study of Rush Alzheimer’s Disease Centre (RUSH-ROS and RUSH-MAP)\textsuperscript{30, 31} were used as replication analysis of rs713224 (see online Supplementary text S1 for the study descriptions and Supplementary text S2 for the items of CES-D scale). With these sample sizes, we had about 80% power to detect associations that explain about 0.12% of the trait variation in the discovery cohort and replication cohort with a \( p \)-value of \( 5 \times 10^{-8} \) and 0.05, respectively. In case of multiple measurements the study centers preferred to analyze the measurements that maximize the number in the analysis. This is usually the first measurement as the response declines by years of follow-up. In this case the mean age of the samples refers to the time of measurement date.

GWAS analyses were performed individually by the study centers, according to the same analysis plan; each study excluded dementia cases (Mini-Mental State Examination score < 22), and anti-depressive medication users (except BLSA), since the effect of anti-depressive medication on the scales was not consistent across the studies. There was no restriction on age. Each study center computed the subscales of the CES-D questionnaire that resulted in four separate scores for each individual, measuring different domains of complaints. The reliability coefficients (Cronbach’s alpha) for the somatic complaints (seven items), lack of positive affect (four items) and negative affect (seven items) domains were adequate and ranged between 0.68 to 0.84 whereas for the interpersonal problems domain (two items) those were between 0.45 to 0.63 for the 20-item CES-D scale cohorts. For the 10-item scale, Cronbach’s alpha’s for the somatic complaints (three items) ranged from 0.52 to 0.78 and for lack of positive affect (two items) and negative affect (three items) between 0.64 and 0.71. Each study implemented linear regression models, adjusted for age, age-square and sex, under the assumption of an additive genetic model, regressing each subscale on allele dosage and reported the summary statistics. The genotyping and imputation methods for each study are given in Supplementary Table S1. Additional study site-specific adjustments included linear mixed-effect models to account for familial correlations in the FHS and ERF, and adjustment for the top three Eigen vectors in RUSH-MAP, RUSH-ROS and STR. Prior to meta-analysis, all single nucleotide polymorphism (SNP) IDs were mapped to dbSNP Build 129. Possible measurement and scoring differences across different study
centers were checked through extracting median standard error from the GWAS summary statistics of each study center and plotting it against the square-root of the sample size. Allele frequencies for all SNPs were compared to HapMap frequencies. Stratified Q-Q plots were generated for minor allele frequency (MAF) and imputation quality strata to assess potential sources of inflation. Meta-analyses were performed using the sample size-weighted method as implemented in METAL software package. Due to poor psychometric properties and differences in the median standard errors across the cohorts we excluded interpersonal problems domain from further analysis. Furthermore, this domain has been criticized for not being consistent with the current criteria for depression and therefore introducing confounding in the validity of the CES-D. SNPs with a MAF less than 2.5% or an observed: expected variance ratio (imputation quality) less than 0.30 were excluded on a per-study basis. SNPs for which the total sample size was lower than 5000 were removed from further analysis. We did not use genomic control as this method has been shown to be too conservative. SNP-based heritability was calculated using 1,069,063 markers that were common in the meta-analyses results and linkage disequilibrium (LD) scores were computed using the 1000 Genomes Central European (CEU) reference panel as suggested by the tutorials and provided by the developers of the method.

To test the amount of variance explained by the genetic risk score, we performed a genetic risk score analysis. We excluded one of the cohorts (RS I) (n=3,709) from the discovery set and used this cohort as the target sample. The total score for individuals was calculated for each set of SNPs that were defined on the basis of the p-values in the discovery set (e.g. p<0.00001, 0.0001, 0.1, 0.2). Genetic risk scores were calculated by multiplying the Z-score that was obtained in the discovery analyses with the risk alleles per SNP (0, 1, 2). The PLINK toolset was used to calculate the risk scores. Linear regression analysis was used to test the association of the genetic risk scores with somatic item scores in the target sample.

RESULTS

The inflation factors for the discovery GWAS of the three scales varied between 1.026 and 0.984. We did not observe any genome-wide significant SNPs for any of the scales in the discovery set apart from the top SNP, rs713224, that showed significant association with somatic complaints scale (p_{discovery} = 3.82 × 10^{-8}). Q-Q plots and Manhattan plots of this analysis are presented in the Supplementary Figs S1 and S2. Supplementary Table S2 shows the SNPs with p<10^{-4} from the discovery set of 32,528 persons for the somatic, positive and negative domains. The analysis of the rs713224 was further extended to a second stage, which included 6,813 persons from five study samples, as shown in Table 1. Study-specific summary results for rs713224 are given in Supplementary Table S3. The overall
analysis yielded a non-significant result ($\rho_{\text{discovery}\&\text{replication}} = 1.10 \times 10^{-6}$) in the genome-wide scale. Testing for heterogeneity showed evidence for outliers ($\rho_{\text{het}} = 0.07$) in the combined analysis, compared with the discovery phase ($\rho_{\text{het}} = 0.17$).

| Table 1 Study sample characteristics of discovery and replication cohorts |
|---------------------------------|--------------|--------------|----------------|--------------|----------------|
|                                 | Somatic items | Negative items | Positive items | Interpersonal items | Age (years) |
| Discovery sample                | Mean(SD)     | Mean(SD)     | Mean(SD)       | Mean(SD)       | Mean(SD)    |
| BLSA                            | 2.92(2.81)   | 1.42(2.41)   | 10.31(2.45)    | 0.22(0.65)     | 71.6(13.8)  | 827   | 45.1  |
| DHS                             | 2.92(3.18)   | 1.54(2.91)   | 7.03(3.25)     | 0.22(0.72)     | 52.4(13.7)  | 991   | 52.6  |
| ERF                             | 3.78(3.76)   | 2.07(3.36)   | 8.43(3.40)     | 0.40(0.89)     | 55.0(10.1)  | 1107  | 55.2  |
| FHS                             | 1.05(0.751)  | 0.61(0.75)   | 0.596(0.739)   | 0.15(0.35)     | 56.1(10.5)  | 6636  | 51.8  |
| HBCS                            | 3.79 (3.31)  | 2.00 (3.05)  | 9.22 (2.40)    | 0.37(0.79)     | 63.4(2.9)   | 1360  | 59.4  |
| HEALTH ABC (Eur)                | 1.68 (2.13)  | 0.93(1.85)   | 10.74(1.82)    | 0.13(0.49)     | 73.8(2.8)   | 1520  | 46.4  |
| RS I                            | 1.52 (2.61)  | 1.24(2.65)   | 10.36(2.59)    | 0.09(0.42)     | 72.7(7.2)   | 3709  | 58.1  |
| RS II                           | 1.98 (2.78)  | 1.34(2.66)   | 10.19(2.60)    | 0.15(0.53)     | 64.8(8.0)   | 1995  | 53.3  |
| RS III                          | 2.66 (3.32)  | 1.17(2.58)   | 10.37(2.41)    | 0.18(0.58)     | 56.0(5.7)   | 1917  | 55.1  |
| SardiNIA                        | 3.27(2.91)   | 2.51(3.08)   | 4.81(2.53)     | 0.48(0.85)     | 58.0(11.4)  | 2608  | 58.1  |
| ARIC<sup>10 item</sup>         | 2.31(2.15)   | 1.19(1.72)   | 5.62(0.95)     | 0.21(0.65)     | 72.7(5.5)   | 384   | 42.1  |
| STR<sup>10 item</sup>           | 1.10(1.60)   | 0.79(1.43)   | 1.22(1.27)     | 0.21(0.58)     | 57.7(8.9)   | 9474  | 52.7  |
| Replication sample              |              |              |                |                |              |
| FINRISK<sup>10 item</sup>      | 1.74(1.63)   | -             | -              | -              | 53.1(13.4)  | 605   | 49.7  |
| HRS<sup>10 item</sup>           | 1.38(1.50)   | -             | -              | -              | 69.3(5.5)   | 3753  | 58.1  |
| InCHIANTI<sup>20 item</sup>    | 3.23(3.18)   | -             | -              | -              | 66.0(15.0)  | 1019  | 47.0  |
| RUSH MAP<sup>10 item</sup>     | 0.50(0.80)   | -             | -              | -              | 80.8(6.5)   | 721   | 71.7  |
| RUSH ROS<sup>10 item</sup>     | 0.57(0.82)   | -             | -              | -              | 75.5(7.2)   | 715   | 65.9  |

Mean; mean value of each scale, SD; Standard deviation of the mean, N; number of subjects included. BLSA; Baltimore Longitudinal Study of Aging; DHS; Dortmund Health Study; ERF; Erasmus Rucphen Family Study; FHS; Framingham Heart Study; HBCS; Helsinki Birth Cohort Study; HEATLH ABC (Eur); Health, Aging and Body Composition study (of European ancestors); RS I-II-III; Rotterdam study first, second and third waves; SardiNIA; SardiNIA study; ARIC; Atherosclerosis Risk in Communities study; STR; Swedish Twin Registry; FINRISK; National FINRISK Study of Finland; RUSH MAP, RUSH Memory and Aging Project; RUSH ROS, RUSH Religious Orders Study.

SNP-based heritability estimates ($h^2$) were 0.038 (SE= 0.01), 0.01 (SE= 0.01) and 0.024 (SE= 0.01) for the somatic, positive, and negative domains, respectively. The somatic and negative domains showed significant co-heritability (genetic correlation= 1.1, SE= 0.23, z-score=4.6, $p= 4.3 \times 10^{-6}$). The positive domain did not show significant genetic correlation with the negative domain (genetic correlation: 1.5, SE= 1.4, z-score= 1.1, $p = 0.27$) or with the somatic domain (genetic correlation: 1.5, SE= 1.3, z-score= 1.1, $p = 0.27$).
In order to search for possible real associations among the subthreshold loci we have performed also a risk score analysis using the discovery set after excluding one of the cohorts as discovery and the RS as the training set. The SNPs with p values less than $10^{-5}$ explained a significant but very small part of the variance on somatic items scale ($p=0.001$, $R^2 = 0.3\%$) (Supplementary Figure S4).

**DISCUSSION**

We conducted a GWAS on specific symptom domains of depression in which we combined the results of 12 population-based studies including 32,528 individuals to find common variants that increase the vulnerability to a particular symptom domain (somatic complaints, lack of positive affect and negative affect). In the discovery set we found evidence for one SNP near the brain-expressed melatonin receptor ($MTNR1A$) gene with respect to somatic complaints domain only ($p_{\text{discovery}} = 3.82 \times 10^{-8}$). This is in line with an earlier study showing that symptoms of depression linked with physiological functions may show higher heritability compared with symptoms related to negative affect $^{36}$. Rs713224 was further analyzed in five separate samples and also in combined meta-analyses of the discovery and replication sets. However, the level of significance of this SNP was attenuated ($p_{\text{discovery+replication}} = 1.10 \times 10^{-6}$). The negative and positive domains did not yield any genome-wide significant SNPs.

Our top SNP, rs713224 is located near the $MTNR1A$ gene, which encodes one of the two melatonin receptors expressed in brain. Melatonin is a circadian and seasonal regulator in many organisms including humans and is secreted in darkness by the pineal gland. Although melatonin is the hormone of the pineal gland, $MTNR1A$ is ubiquitously expressed, predominantly in suprachiasmatic nucleus, hypothalamus and prefrontal cortex. The melatonin receptor pathway is known to be involved in depression $^{37-42}$ and its relationship with somatic complaints, and vitality in general, makes it a biologically plausible gene.

However, lack of replication raises the conclusion that our finding for this SNP is likely to be a false positive. Among other reasons, population stratification can result in false-positive findings. To avoid population-stratification, only individuals of European descent were included in this study. Including only individuals from population-based studies of European descent also minimized measurement error caused by cultural differences in response to the CES-D $^{43}$. On the other hand, this does not count for the replication set. Among them, for instance RUSH sample included more included more women and older persons and reported very low mean values for the somatic items (Table 1). Difference in age across the study samples can introduce heterogeneity since melatonin and melatonin
receptors are shown to decrease by age \(^{44}\). An additional sensitivity analysis, excluding the RUSH study, yielded a \(p_{\text{discovery+replication}} = 4.96 \times 10^{-7}\) and decreased the heterogeneity \(p_{\text{het}} = 0.08\), but did not exclude it completely. A particular reason of heterogeneity for the melatonin receptor signaling-related outcomes is the interaction with melatonin levels as reviewed extensively before \(^{45}\) and therefore the influence on depressive symptoms maybe season specific, depending on the calendar time and latitude that the depression screening took place. However, it was not possible to control for this in current study.

Although our study is among the largest ones conducted thus far on the common genomic variation in depression with power to detect effects explaining 0.12% of the variation, our study failed to clearly detect and replicate a single loci related to symptoms of depression. Among several reasons one is that the trait may not be genetically controlled.

The CES-D questionnaire measures depressive symptoms in the past week and the total CES-D scale has been shown to be conserved through life \(^{11}\), while this does not rule out the fact that responses to different symptom clusters may differ throughout lifetime as there is no study to our knowledge has focused on this. In addition, response to specific symptom clusters may be population specific due to cultural acceptance or practices. Moreover, age differences across the CHARGE cohorts might have played a role as presentation of depressive symptoms strongly differs by age whereas some genetic variants hypothesized to interact with age \(^{46}\). These would probably introduce phenotypic and genetic heterogeneity. To see if the individual studies were indeed genetically controlled we estimated the SNP-based heritability from the separate GWAS summary statistics collected in this research. The SNP-based heritability estimations in the meta-analysis were surprisingly low (1-4%), indicating the reason for sparse findings. This is partially due to several reasons sourcing from the genetic architecture of the traits, which are not adequately addressed by the simple association models; such as exclusion of X chromosome, and limiting the analysis only with additive genetic model which deviates from sufficient power when the MAF < 0.5. Another important reason is that interaction of any genetic determinant with stressful life events, traumas, therapeutic agents, smoking or menopause, which may confer risk to depression \(^{47}\), were neglected. We further estimated the co-heritability to see if there are genetic outliers amongst the meta-analysis cohorts. This revealed surprisingly low co-heritability across the contributing cohorts, explaining the lack of successful meta-analysis and replication in our study. The low co-heritability estimations are the indicators of high genetic and phenotypic heterogeneity across the cohorts and are the plausible explanation of insignificant replication in our research. Here it is important to note that the estimates from LD score regression have to be treated with caution because of the small sample size in some individual cohorts.
We also considered the possibility that individual common SNPs explain only very small proportion of some complex traits, as shown by the polygenic risk score analyses in the current study in which only 0.3% of variance was explained by the most significant SNPs. The risk score for the remaining thresholds did not improve the explained variance, contrary of previous reports. Previous studies exploring complex traits (e.g. educational attainment, MDD) revealed similar results.

The difficulty in finding and replicating GWAS signals for major depression has been a common experience both for depressive symptoms and MDD. A previous study of depressive symptoms of the CHARGE Consortium on a partially overlapping sample suggested a region on 5q21 in a combined analysis of more than 50,000 persons. A meta-analysis of eight GWAS of MDD status (about 6,000 MDD cases and about 7,000 controls), yielded only one genome-wide significant finding in the solute carrier family 6 member 15 gene (SLC6A15), while the recent Psychiatric Genomic Consortium (PGC) mega-analysis (9,238 cases and 8,039 controls) pointed out one region on 3p21.1 that reached genome–wide significance; however to our knowledge no replication has been reported so far. PGC-MDD GWAS also showed association of rs4478239, located within 800 kb of MTNR1A, with recurrent depression ($p = 4.7 \times 10^{-7}$) in a study including 6743 cases and 9519 controls (Supplementary Fig. S3). However, the proxy for our top SNP in that region (rs2375800) was not associated with MDD. Similarly we were not able to replicate the two main findings of a recent report by the CONVERGE Consortium who attributed the success to the recruitment of relatively homogeneous cases with severe illness. For the LHPP gene region the proxy SNP rs12258489 yielded insignificant $p$-values for negative items ($z$-score = 1.52, $p = 0.12$), for positive items ($z$-score = -0.52, $p = 0.59$) and for somatic items ($z$-score = 1.23, $p = 0.22$). For the sirtuin 1 (SIRT1) region, the SNP of interest rs12415800 did not associate with negative items, ($z$-score = 0.12, $p = 0.89$), positive items ($z$-score = -0.57, $p = 0.56$) or somatic items scales ($z$-score = 1.81, $p = 0.07$).

To conclude, our efforts in a large collaboration utilizing phenotypes defined by symptom clustering yielded no genome-wide significant hit except the somatic complaints domain. One SNP, associated with somatic complaints, reached genome-wide significance in the combined sample and suggested the involvement of MTNR1A in the melatonin signaling pathway, but was not further replicated. Our results suggest that GWAS for depression in large population-based samples remain underpowered due to phenotypic and genetic heterogeneity.
REFERENCES


Chapter 4.2

A. Somatic items scale  
B. Positive items Scale  
C. Negative items scale  

**Supplementary Figure S1.** Q-Q plots of the overall meta-GWAS
A genome-wide association study of the CES-D domains

A. Somatic Items Scale.

B. Positive Items Scale.

C. Negative Items Scale.

Supplementary Figure S2. Manhattan plots of the overall meta-GWAS
**Supplementary Figure S3.** *MTNR1A* in the PGC-MDD results
Supplementary Figure 4. Percentage of variance explained by genetic risk scores in the Rotterdam Study.
* $p < 0.05$
Chapter 5

Discussion
DISCUSSION

5.1 Rationale

In this thesis, I investigated hormonal, vascular and genetic determinants of the most common mental health disorder, depression. These determinants have been examined previously in clinical and epidemiological studies. In the studies presented I attempt to advance our knowledge by exploring hormonal, vascular and genetic risk factors in a population-based sample including milder and more severe forms of depression. Also, we used various methodological approaches to explore the associations more in detail.

In the current chapter, I will discuss the main findings of the studies included in this thesis. Also, I will discuss methodological challenges encountered in the studies. Additionally, I will summarize clinical implications of the findings and future directions for research.

5.2 Main findings

Epidemiological studies of the HPA axis

Dysfunctional stress response is a key finding of several chronic diseases. Intense investigations have been made to elucidate the effects of the dysfunctional stress response on the development of chronic diseases. However, these studies were mostly performed in small clinical samples. The number of large scale epidemiological studies is limited and evolved only in the last decades with the advances in biological sample collection and measurement. Measurement of diurnal cortisol rhythm is not feasible in blood or urine samples and more valid assessments require repeated sampling over the day. Therefore, progress on saliva assessments of cortisol made it possible to collect several saliva samples without any professional assistance. Also, modifications in the dose of dexamethasone in the suppression test and the assessment of the cortisol response in saliva facilitates HPA axis suppression research in epidemiological studies. In the last decade, large-scale cohort studies published the result of cortisol research of psychiatric traits and other chronic diseases which provided novel information about this biological marker of many chronic diseases. These epidemiological studies generally assess many exposures and outcomes that make systematic assessment of cortisol levels particularly valuable. Further, these large-scale studies often include genetic data providing a great opportunity to explore common genetic variants in relation to HPA axis activity as measured by cortisol.

In chapter 2.1, we explored possible effects of smoking, a common risk factor for mental and physical diseases, on the HPA axis to elucidate a possible pathway to chronic diseases. We found that smoking was independently associated with hypercortisolemia throughout the day and a higher morning increase of cortisol. Also, we found that effects of smoking
on the HPA axis were temporary as there were no difference in terms of cortisol measures between former and never smokers. Moreover, time since quitting was associated with a decline in the average daytime cortisol levels.

In accordance with our study of older adults, high cortisol levels and increased cortisol awakening response in younger smokers were reported previously. The cortisol awakening response, which is related to hippocampal networks controlling memory and orientation and preparing individuals to the demands of the upcoming day. Smoking is a physiological stressor that might activate the HPA axis directly. Also, smokers might involve more stressful life events or perceive more stress compared to non-smokers which might explain high cortisol levels and increased CAR.

One of the most important finding of our work was showing the temporary effect of smoking on cortisol measurements. We know that many chronic disease risks decrease after smoking cessation if no permanent changes occurred in the body. We showed one of the mechanisms underlying increased HPA axis activity.

In chapter 2.2, we evaluated the determinants of the negative feedback of the HPA axis using a very low-dose (0.25 mg) dexamethasone suppression test. Being female, low income, lack of exercise, instrumental disability and smoking were related to a strong suppression of the HPA axis as measured by low cortisol levels after dexamethasone intake. However, clinically relevant depressive symptoms and anxiety disorders were more prevalent in people with a non-suppression of cortisol as measured by high cortisol levels after dexamethasone intake. Determinants of the negative feedback in general population have been explored in the NESDA study. The authors found that smoking, low daily activity and sampling on a weekday were related to a non-suppression of cortisol after dexamethasone intake. However, these studies are not truly comparable as in the NESDA study the authors used a higher dose of dexamethasone, which suppressed the HPA axis in almost 87% of the study population. In our study, we evaluated cortisol levels continuously to use all possible information and detect subtle differences. Also, we used a cut-off to define non-suppressors only to test non-linear associations of psychiatric traits, even though defining an arbitrary cut-off is not the optimal way to test such associations as it might be misleading. Our results suggest a non-linear association between non-suppression of the HPA-axis and depression and anxiety in the general-population.

As mentioned above, in this chapter, we aimed to uncover pathophysiological pathways underlying the association of cortisol and chronic diseases. In chapter 2.3 the genetics basis of plasma cortisol was explored. Plasma cortisol is a heritable (30-60%) trait. To date, only small candidate gene studies have been done to identify genetic variants of
plasma cortisol. In this study, we performed a GWAS on morning plasma cortisol in 12,597 people. We found and replicated common genetic variants at chromosome 14q32 which contains the SERPINA6 and SERPINA1 gene. We explored also the functional consequences of these genetic variants and found that they affect plasma cortisol levels as they alter the level cortisol binding globulin (CBG). Additionally, we found that the top hit affect the cleavage of the CBG from the reactive center loop. It is known that the cleavage decreases the cortisol binding affinity of the CBG resulting in higher levels of circulating cortisol. In this large GWA study, we have shown the genetic variants related to the plasma cortisol as well as their biological effects.

In chapter 2.4, we explored the SNP-based heritability of single time point measure of plasma and saliva cortisol levels. We used the data of plasma cortisol GWAS that was presented in Chapter 2.4. Additionally, we performed a GWAS using morning saliva cortisol levels in 7703 participants in eight European cohorts and found no genome-wide significant hit. We detected low SNP-based heritability of plasma and saliva cortisol levels. A Single time point of a cortisol assessment has large intra-individual variability that is affected by various environmental factors. Therefore, it can be expected to have low variability to be explained by genetic markers as they can be considered as a state-marker rather than a trait. Using a more stable marker of cortisol secretion such as area under the curve using consecutive days, hair cortisol that are not free-of environmental factors might be more useful to calculate SNP-based heritability.

Vascular depression hypothesis in epidemiological studies
Cerebral small vessel disease is a term that can be conceptualized with pathological, clinical and neuroimaging findings. The clinical presentation ranges from no to severe neurological symptoms and disability. With the advances in neuroimaging techniques, it is now possible to detect non-clinical cerebral small vessel disease. This created the opportunity to test the vascular depression hypothesis.

In chapter 3, we aimed to scrutinize the association of several neuroimaging indicators of cerebral small vessel disease with depression in cross-sectional and longitudinal analyses in community-dwelling subjects.

Walking down memory lane, in chapter 3.1, we revisit white matter lesions and silent brain infarcts in relation to depression. Here, we aimed to establish the longitudinal associations of white matter lesions and silent brain infarcts with depressive disorders in 1047 non-demented participants. Severe subcortical white matter lesions increased the risk of incident depression. Asymptomatic brain infarcts were related with almost three-fold increased risk of a recurrent depressive episode during a mean follow-up of 3.6 years. In
more recent work presented in chapter 3.2. we tested several imaging markers of cerebral small vessel disease and different severity degrees of depression in 3799 participants cross-sectionally. White matter lesion volume and lacunar infarcts were related to depressive symptoms and disorders. Cerebral microbleeds were associated to depressive disorders only. This association was seen particularly pronounced in persons with depression and comorbid anxiety, which indicate a more severe form of depression. Deep/infratentorial cerebral microbleeds were found more often in persons with depressive disorders, whereas no association was detected between lobar microbleeds and depressive disorders.

The high prevalence of white matter lesions in people with depression is one of the earliest findings supporting vascular depression hypothesis. We detected a cross-sectional association between white matter lesion volume and depression. Also, we found that presence of severe white matter lesions doubled the risk of incident depression in longitudinal analyses. Overall our results are consistent with previous studies. Similarly, silent lacunar infarcts have been related to depression especially in late life which was replicated in cross-sectional and longitudinal studies presented in this chapter.

According to Taylor et al. there are several hypotheses which explain the association between white matter lesions or silent infarcts and depression. First, the group proposed the threshold model, which posits that more severe vascular burden in the white matter of brain regions regulating mood such as frontolimbic tracts would increase vulnerability to depression. After a certain threshold of cerebrovascular burden is exceeded, symptoms of depression emerge. Second, they defined the disconnection hypothesis, which posits that focal lesions in specific tracts related to mood regulation are more important than global vascular burden. In our studies, we did not evaluate the locations of the lesions in detail. But we found that lesions located in subcortical regions, which includes regions regulating mood, are related to incident depression rather than the periventricular lesions.

Similar to white matter lesions and lacunar infarcts, cerebral microbleeds are the silent lesions of cerebral small vessel disease. They are small hemorrhages occurring as a result of structural vascular damage in small cerebral arteries that are common in elderly. They have been related to stroke and post-stroke depression. However, it has not been tested whether there is a relationship between cerebral microbleeds and depression that is independent of stroke. In our study, we observed an association between cerebral microbleeds and major depressive disorder in stroke-free participants. Interestingly, the association with depression was significant for deep/infratentorial cerebral microbleeds only. According to the literature, deep/infratentorial cerebral microbleeds are related to cerebrovascular risk factors and hypertension, whereas strictly lobar microbleeds are related to cerebral amyloid angiopathy. Overall, our findings further the knowledge on vascular depression hypothesis.
showing the association of cerebral microbleeds with depressive disorders as these lesions differs from white matter lesions and lacunar infarcts. Additionally, findings on the location of the microbleeds are in favor of vascular etiology of depression.

In the light of the studies in this chapter and previous publications, we postulate that white matter lesions and lacunar infarcts might be an unspecific marker of vascular lesions in depression. Ischemia is an important underlying pathology behind white matter lesions and silent infarcts. However, ischemia itself is not a specific pathological mechanism as it is a result of several vascular pathologies from endothelial dysfunction to cerebral hemodynamics. A constant cerebral blood flow is needed for cerebral hemostasis. Alterations of the cerebral blood flow or defects in the autoregulation of cerebral blood flow can cause vascular lesions many years before the clinical onset of a cerebrovascular disease. Also, cerebral perfusion deficits may affect protein synthesis important for cognitive networks and synaptic plasticity.

Cerebral blood flow velocity and vasomotor reactivity are two important dynamic measures of cerebral vasculature. While cerebral blood flow velocity is considered as an indicator of cerebral metabolism, vasomotor reactivity is an indicator of microangiopathy reflecting autonomous disturbances in the cerebral vessels. In Chapter 3.3, we explored the link of cerebral blood flow velocity and vasomotor reactivity with clinical and subthreshold depression in 1494 elderly people. We found that low mean cerebral blood flow velocity at baseline were related to higher depressive symptom scores at follow-up. Mean cerebral blood flow velocity predicted incident depressive symptoms and depressive disorders. Low baseline vasomotor reactivity predicted depressive disorders only. Our results are in line with the results of cross-sectional studies. Here, we furthered the evidence showing a temporal association between cerebral hemodynamics and incident depression in people free of cerebrovascular diseases. In this study, analyses were controlled for several vascular risk factors, cerebral atherosclerosis, indicators of poor health, and stroke during follow-up and results did not change substantially. Therefore, we can conclude that early changes in cerebrovascular hemodynamics predict depression supporting vascular depression hypothesis.

The co-occurrence of depression and dementia is well-known from clinical and non-clinical studies. To date, it is still not clear if they share the same etiology or depression is a risk of dementia or both. We know that depression in late life is related to cognitive disturbances especially when vascular risk factors are present. Many studies explored vascular risk factors to explain the association between depression and dementia. However, there is not only a large body of evidence implicating vascular pathology in Alzheimer’s disease but also amyloid pathology. In Chapter 3.4, we explored amyloid pathology in persons
with depression taking prodromal dementia into account. Previous studies on Aβ and depression were mostly cross-sectional and yielded conflicting results \(^{32-39}\). We assessed the association of plasma amyloid beta (Aβ) levels with depressive syndromes in a cohort of 980 dementia-free people. In cross-sectional analyses, we found that high levels of Aβ\(_{1-40}\) were related to clinically relevant depressive symptoms. However, this association was explained by prodromal dementia. In prospective analyses, we found that low Aβ\(_{1-40}\) and Aβ\(_{1-42}\) levels were related to a higher risk of incident clinically relevant depressive symptoms at follow-up in people with no dementia prodrome during the follow-up. In these analyses, persons who developed dementia during the maximum follow-up of 13 years were excluded. Therefore, it is unlikely that these results were driven by dementia prodrome. Cerebral and peripheral amyloid levels are in a dynamic equilibrium. When amyloid peptides accumulate in the brain, peripheral levels are decreased \(^{40}\). An Aβ accumulation in the brain can well be related by lower circulating levels in peripheral blood. In these analyses, we controlled for common vascular risk factors and diseases and results did not change substantially. However, we cannot rule out that non-overt cerebrovascular disturbances contributed to the associations as we could not control for white matter lesions, cerebral microbleeds, or cerebrovascular hemodynamics. Cerebral small vessel disease is related to perivascular Aβ accumulation and transient Aβ deposition in the brain causing alterations in cerebral perfusion and is neurotoxic for monoaminergic neurons \(^{31,41-44}\). Therefore, it is still possible that the association of low Aβ levels with incident depression in dementia-free persons is related to underlying vascular pathology.

**Depression and genetic epidemiological studies**

One of the most striking developments in epidemiology in the last decades is the development of GWAS. Many novel genetic variations have been detected in GWAS of different phenotypes in psychiatry. However, genetic research on depression, although a heritable phenotype, was no success story – even when using a GWA approach. In Chapter 4, we aimed to explore the genetic variants related to depression using different perspectives of methodologies and phenotype definitions.

In chapter 4.1, we combined two previously published GWAS studies in which depressive symptoms and depressive disorders were evaluated separately. We found and replicated one locus related to the broad depression phenotype located in the intron of the FHIT gene that is related to circadian clock modification, oxidative stress, and the level of DNA-damage. This is the first locus detected in a GWAS study of the broad depression phenotype of people of European ancestry. One of the most problematic aspects of GWAS in depression is the lack of statistical power for such heterogeneous phenotype. Using a more inclusive approach helped us to increase the statistical power.
In chapter 4.2, we assessed the genetic variants that are related to depressive symptom clusters based on the CES-D scale. One common variant located in the brain-expressed melatonin receptor (MTNR1A) gene was related to the somatic symptoms. The melatonin pathway is well-known for depression. As somatic symptoms include sleep, appetite and fatigue, it is a very plausible gene for this symptom cluster. However, this variant was not replicated. Even though we aimed to harmonize depression phenotype, our study was still underpowered.

5.3 Methodological considerations

Cortisol assessment in large population-based studies

Cortisol follows a diurnal rhythm increasing with awakening, maximizing approximately 30 minutes after awakening and then decreases during the day. Therefore, a single measurement of cortisol levels is generally not informative about the HPA-axis function. However, repeated plasma measurements are not feasible especially in population-based studies. Cortisol can be measured in saliva with sampling devices that can be applied by individuals at home requiring no special assistance. This was the catalyst of cortisol research in large epidemiological studies in the last decade. In the Rotterdam Study, saliva samples were used to assess cortisol levels. This large data collection enabled us to evaluate cortisol levels over the day and to calculate important summary measures indicating diurnal cortisol rhythm.

Negative feedback control of the HPA-axis is an important and distinct function that requires dexamethasone suppression test in which participants need to take a dexamethasone pill. However, interventional methods, especially the classical DST which requires 1 mg dexamethasone intake, are almost impossible to use in population-based studies as this dose might be too high for general population. Such high dose may suppress the HPA-axis and does not let to evaluate the negative feedback of the axis as most of the subjects would have a fully-suppressed HPA-axis. Therefore, using a very low dose dexamethasone (0.25 mg) let us to evaluate several determinants of the negative-feedback of the HPA-axis. Overall, applying such an invasive method and assessing cortisol in saliva in a large-scale, population-based study with an extensive data collection was helpful to consider several covariates and to assess many determinants of the HPA-axis which is not possible in a small, clinical study.

HPA-axis studies in large population-based studies generally suffer from non-compliance to the sampling protocol and ingestion of dexamethasone. Evaluating the dexamethasone levels would be helpful to assess the compliance to the dexamethasone ingestion. In the Rotterdam Study, this was not feasible as we used a very-low dose of dexamethasone.
However, non-compliance of dexamethasone ingestion does not seem to be common in studies 4, 45. Accurate time of awakening and time of sampling are vital to evaluate the cortisol awakening response. Electronical monitors can be used to obtain accurate time of sampling but these devices are costly for large scale studies. Most of the epidemiological studies give clear instructions emphasizing the importance of actual sampling time. Studies have shown that participants report their awakening and sampling times accurately 46. In the Rotterdam Study, all participants are given clear instructions about time of sampling. When we excluded participants with poor compliance to the DST protocol, we did not observe a meaningful change of the effect estimates. As our definition of poor compliance relies on the self-report data, there is still a risk of residual information bias.

**Phenotype definitions in epidemiological studies**

Depression is phenotypically heterogeneous psychiatric disease including affective, somatic, and cognitive symptoms. In medicine, it is common practice to diagnose a disease on the basis of symptoms only. Yet, no other discipline completely relies on observation and symptoms report only. Different combinations of several symptoms can yield the same diagnosis in psychiatry and other disciplines. Consequently, many diseases have the problem of heterogeneity to a certain degree. However, the heterogeneity problem is more prominent in psychiatric phenotypes because of weak measurement tools, lack of accurate and precise biomarkers, non-empirical and convention-based categorical definitions, and unreliable subtype definitions. To make the matter worse, both a decreased or increased levels of behavioral symptoms like appetite, sleep and activity level are considered symptoms of depression. Including both extremes of symptoms is unusual in a single non-psychiatric disease entity.

Classification systems are very important in medicine in general, as they are required for treatment decisions. Psychiatric diagnoses are made using the prevailing diagnostic systems like DSM or ICD. However, such approaches obscure both the variations within the disease and similarities between the diseases. This has emerged as a great problem in psychiatry research.

A growing body of evidence indicate that depression is better conceptualized, measured and even categorized (using convention-based cut-offs) better when dimensional models are used. Evidence supporting the depression continuum is derived from statistical and epidemiological studies. Statistical studies indicate that latent structure of depression has no discontinuities as defined in DSM or ICD and severity of depression is continuously distributed. Also, dimensional models of depression showed better predictive validity than the DSM-based categorical models 47-49. In longitudinal epidemiological studies, it was repeatedly shown that milder forms of depression such as subthreshold depression or minor
depression increase the risk of subsequent major depressive episodes. \(^{50-52}\) Also, milder forms of depression or greater numbers of depressive symptoms have been related to increased mortality, disability and worse life quality \(^{52, 53}\).

Dichotomization of the diseases on the basis of classification systems is used mostly for efficacy and to enhance the contrast in non-clinical research. In the studies presented in this thesis, we studied depressive symptom scores continuously and categorically using a cut-off score to define clinically relevant depressive symptoms. Among persons with clinically relevant depressive symptoms, we further defined different forms of clinical depression to evaluate biological characteristics separately. Subthreshold depression was defined as a combination of depressive symptoms including at least one of the cardinal symptoms of MDD, however, not meeting DSM-IV MDD criteria for duration, impairment or number of symptoms. Additionally, we considered depressive episodes comorbid with anxiety disorders as a more severe form of depression. This approach helped us explain and detect indicators of severe MDD as well as less severe forms of depression in Chapter 3. Enhancing the contrast using categories made it possible to assess the probability and risk of having less and more severe types of clinical depression compared to a reference group in a very straightforward way (e.g. using odds ratio estimated) even though that comes with a risk of misclassification and multiple testing yielding false-positive results.

One of the obstacles to be overcome by GWAS studies in depression is the lack of sufficient power to detect common variants with small effect sizes. Therefore, any attempt to increase the sample size in GWAS is welcome. Following this reasoning and a known correlation between depressive disorders and depressive symptoms, we pooled the results of two largest GWAS of life-time depressive disorders and depressive symptoms. Using this approach, we achieved to collect genetic data from one of the largest meta-analyses in a GWAS in psychiatry to date at the risk of having substantial phenotypic heterogeneity which would most likely lead to an underestimation of the effects. However, it must be admitted that increasing sample size using well-defined subgroups is more efficient for a GWAS as most of the information on a continuous depressive symptoms score is less informative if the population has very low scores.

In the depression GWAS, we combined two GWAS-meta-analyses in which categorical and continuous traits were used. We used a p-value based meta-analysis approach implemented in the program METAL. In this method, Z-scores for each allele is calculated and combined across samples using weights. Weights are proportional to the square root of the sample size \(^{54}\). Choosing an optimal weight to obtain sufficient power is challenging as each method has its own particular pros and cons \(^{55}\). However, using the overall sample sizes for two meta-analyses in which categorical and continuous traits were used could cause
inconvenient weighting as the data from the CHARGE consortium had a larger sample size with a continuous trait than in the PGC MDD GWAS. In that kind of situations, performing a series of sensitivity analysis is most helpful. We therefore tested the change in our results with sensitivity analyses using equal weights, weights derived from effective sample size, using proportional sample sizes, and finally using no weights as suggested by Stouffer. Results did not change substantially with different weightings. This approach can be useful when pooling GWAS results with slightly different phenotypes to perform a meta-analysis.

**Longitudinal data and interpreting the results**

Longitudinal studies are the best designs to test causal associations even though there is no clear definition of criteria for causality. Temporality is a prerequisite for causality even though it is not very straightforward to assess temporality in association studies of diseases with a non-clinical prodrome as is the case for many chronic diseases including dementia, schizophrenia, atherosclerosis, cancer, or for any episodic diseases. In these conditions, exposure might seem to precede overt disease, therefore appearing as a causal factor for the disease. In fact, the prodrome or a previous episode of a disease of interest might give rise to the exposure, a phenomenon that is termed reverse causality. In chapter 3.4., we tested whether the association between amyloid beta levels and incident depression was dependent on depressive episodes that occurred before the exposure assessments to assess reverse causality (Figure 1).

\[ \text{History of depression} \rightarrow \text{Amyloid beta levels (Baseline)} \rightarrow \text{Depression (Follow-up)} \]

**Figure 1.** Reverse causality that history of depression causes alterations on amyloid beta levels

In chapter 3.4. we used longitudinal dementia data in cross-sectional analyses to define participants with depression and dementia prodrome as a subtype of depression. The terms used for a study design and the data collection are often confused. For example, prospective (i.e., more recent) data in a cross-sectional study nested in a cohort can be used which was done in chapter 3.4 to define the outcome (i.e. Depression with prodromal dementia) better.

**5.4 Recommendations**

**Clinical implications**

In this thesis, I explored behavioral, biological and genetic determinants of the stress response and depression to unravel etiology and the risk factors using epidemiological approaches in a non-clinical population. As expected, implementing the conclusions made
in this thesis directly to the clinical practice is not easily possible. Nevertheless, the findings invite some clinical reflections.

Smoking is one of the most important risk factors of chronic cardiovascular and cerebrovascular diseases. Smoking cessation, if irreversible changes did not happen, decreases the abovementioned risks rapidly. In this thesis, I have found that smoking cessation is related to lower cortisol levels, which was related to time since quitting. The results suggest that the disturbed stress response, which contributes to the development of chronic vascular diseases on smokers, is reversible. This finding would be helpful to promote smoking cessation programs providing biological evidence for an effect of stopping with smoking.

In this thesis, I broadly explored the vascular depression hypothesis, which was described first as a clinical subtype of depression in spite of the fact that it is now a more research than a clinical entity. I found that early vascular changes such as non-clinical white matter lesions, silent brain infarcts, cerebral microbleeds, low blood flow velocity, and decreased vasomotor reactivity of the cerebral vessels, measured before the disabling symptomatology occurs, increase the risk of incident depression and the recurrence of depression. Overall, these findings indicate that preserving vascular health and prevention of vascular diseases are needed to be implemented in the prevention programs of depressive disorders occurring in later-life.

The genetic studies presented in this thesis show some novel genes and functions about depression etiology and functions of the HPA-axis. In general, results of a GWAS cannot be easily implemented in clinical setting as the predictive power of a SNP for a certain disease is generally low. However, these studies provide data to understand the overall genetic vulnerability of a person and the genetic correlation of different diseases. Personalized genetic liability might have an impact on changing behavior to decrease preventable risk factors. Detecting genetic overlap between diseases using polygenic risk score methods would be helpful to understand the etiology of psychiatric disorders. Also, this approach could provoke a paradigm shift in psychiatric nosology as more and more genetic studies show strong genetic overlap between psychiatric diseases.

**Future directions for research**
Acute and chronic stress response are interconnected yet distinct entities of the stress response. Repeated saliva sampling over the day and even on sequential days is informative about the general activity of the HPA-axis, only. Acute stress response can be evaluated using psychological or physiological stress tests that are burdensome for large scale studies. Easier or even modified methods are required. To assess chronic cortisol secretion, a relatively novel method, hair cortisol, can be used in large population-based studies.
In terms of genetics of the HPA-axis, polygenic risk score analyses can be used to assess whether common risk variants may explain the relationship of cortisol levels with psychiatric traits such as depression, bipolar disorder, psychotic disorders, anxiety disorders, and cognitive problems. Also, more stable markers of the HPA-axis such as hair cortisol, repeated sampling of cortisol in consecutive days might be more useful to detect SNP-based heritability of cortisol. Such traits might be helpful to detect novel SNPs if used in a GWAS as they will be less prone to the environmental factors and noise than single-point saliva cortisol levels.

The vascular depression hypothesis has been tested repeatedly in studies of large vessel diseases. Such an approach does not allow researchers to disentangle direct biological from psychological effects of vascular disease in depressed persons. To overcome this issue, many others and we used non-clinical vascular changes as an exposure measure. Further studies are needed to explore the microcirculation and markers of vascular integrity in depression. Also, longitudinal study design is needed to detect early changes of the vascular status in healthy people to predict subsequent depression. Further, assessment of polygenic risk of vascular pathologies and imaging findings and depression would add another dimension to the vascular depression hypothesis.

In this thesis, I presented different approaches to detect polymorphisms in complex traits. It is obvious that large sample size is required when studying heterogeneous traits. Another lesson taken from the studies was to focus on more homogeneous traits. Therefore, future research on GWAS will be determined by large collaborations with extensive data collections which allow studies of more homogenous phenotypes.

Novel methods using summary statistics of the GWAS now allow to examine the explained variance by a polygenic risk of a certain trait. This type of research summarizes the total effect of common variants with small effects (imprecisely) indicating the individual risk and also common genetic variation between different phenotypes. Also, the SNP-based heritability can be calculated now. Therefore, any new GWAS with better phenotyping and large sample size would help to increase the precision of the risk calculations and would be to even better estimate the shared genetic etiology between diseases.

To date, many genes have been detected for psychiatric traits. However, each variant is different in terms of the allele frequency and the effect size. We still do not know how much overall variance of a disease is explained by genetic variations when we combine all data. Further methodological studies would help to disentangle this issue.
5.5 Concluding remarks

The whole process of this thesis and my clinical observations of depressed patients made me to believe that the biological determinants of depression should be explored more in detail especially in older adults. Even though vascular depression hypothesis has been examined repeatedly, there are still many questions to be answered. I conclude that subclinical cerebral vascular pathologies rather than peripheral indicators play an important role in etiology and progress of depression. Also, more powerful genetic studies of depression would reveal novel genes that would lead to discover or disentangle biological mechanisms playing a role in depression etiology. It is good to know that at the time of writing of this thesis, these studies were on their way and have been successful. Moreover, being part of these studies Being part of these further studies was a which I have contributed. Moreover, it was a privilege to be able to contribute to this endeavor.
REFERENCES


Chapter 6

Summary
SUMMARY

Understanding the biology behind depression has a long history in research. To date, no unique marker or specific etiological factor was detected. However, we know more about the possible mechanisms that predispose depression or affect clinical presentation and prognosis of depression (Chapter 1).

In chapter 2, I aimed to scrutinize the determinants and the genetic control of different functions of the HPA-axis, a well-known neurohormonal correlate of depression. In chapter 2.1, I explored the effect of smoking on daily cortisol to understand how smoking contributes on health via stress-response. I found that smoking is related with hypercortisolemia and quitting smoking reverses the increased cortisol secretion. Chapter 2.2 presents determinants of the negative feed-back function of the HPA-axis that was measured using a very low dose (0.25 mg) dexamethasone suppression test. In this study, I found that being female, low income, lack of exercise, instrumental disability and smoking were related to low cortisol levels after dexamethasone intake. However, clinically relevant depressive symptoms and anxiety disorders were related with a non-suppression of cortisol after dexamethasone intake. In chapter 2.3, I explored common variants related to plasma cortisol levels using a genome-wide association approach. In this study, I found and replicated common genetic variants at chromosome 14q32 which contains SERPINA6 and SERPINA1. We also explored the functional consequences of these genetic variants and found that they affect plasma cortisol levels via the cortisol binding globulin (CBG). Also, we showed some evidence of the CBG cleavage which was activated by the reactive centre loop. In chapter 2.4 we performed a genome-wide analysis of morning saliva cortisol level and measured the SNP-based heritability of saliva and plasma cortisol levels. We found no genome-wide significant association between autosomal SNPs and morning saliva cortisol level. Also, we detected low SNP-based heritability of single-point plasma and saliva cortisol levels.

The potential vascular etiology of depression has been discussed for a long time and has been studied repeatedly. However, studies are mostly clinical with small sample sizes. In chapter 3, I revisited the vascular depression hypothesis and explored the associations of non-clinical cerebrovascular alterations with depression. In chapter 3.1, I found that silent brain infarcts are related with the risk of depression recurrence, whereas subcortical white matter lesions are related to incident depression in non-demented elderly people. In chapter 3.2, I used several neuroimaging markers to test the associations with depression. I found that white matter lesion volume and lacunar infarcts were related to different severity degrees of depression whereas cerebral microbleeds were related with severe depression only. In chapter 3.3 relationship between cerebral blood flow, vasomotor reactivity
and depression were assessed longitudinally. Low mean blood flow velocity at baseline was associated with depressive symptoms and disorder longitudinally. Also, decreased vasomotor reactivity was related to incident depressive disorders in people with no-dementia and no-stroke. In chapter 3.4 we evaluated the association of plasma amyloid beta (Aβ) levels with depressive syndromes in dementia-free people. We found that high levels of Aβ1-40 were related to clinically relevant depressive symptoms cross-sectionally and this association was explained by prodromal dementia. In longitudinal analyses, we found that low Aβ1-40 and Aβ1-42 levels were related to a high risk of incident clinically relevant depressive symptoms in people with no dementia prodrome during the follow-up.

Chapter 4 reports the results of genome-wide association studies in depression. In chapter 4.1 I present the results of two previously published genome-wide association studies that were performed in depressive symptoms and disorders separately. I found and replicated a locus located in the FHIT gene that is related to circadian clock, oxidative stress and the DNA-damage. In chapter 4.2 I assessed common genetic variants in subdomains of the CES-D scale that is consisted of negative affect, positive affect, somatic symptoms and interpersonal relationships. One variant in the brain-expressed melatonin receptor (MTNR1A) gene was related to the somatic symptoms. The melatonin pathway is well-known for depression. As somatic symptoms include sleep, appetite and fatigue, it is a very plausible gene for this symptom cluster. However, this variant was not replicated.

In chapter 5, I discussed the most important findings of the studies and summarized methodological issues and clinical implications.
SAMENVATTING

Het begrijpen van het biologie mechanisme achter depressie heeft een lange geschiedenis in het onderzoek. Tot op heden is geen unieke marker of specifieke etiologische factor gedetecteerd. We weten echter wel meer over de mogelijke mechanismen die voor depressie predisponeren of de klinische presentatie en prognose van depressie beïnvloeden (hoofdstuk 1).

In hoofdstuk 2 heb ik kritisch de determinanten en genetische controle van verschillende functies van de HPA-as bestudeerd, wat een bekende neuro hormonale correlatie van depressie is. In hoofdstuk 2.1 onderzocht ik het effect van roken op dagelijkse cortisol om te begrijpen hoe roken bijdraagt aan de gezondheid via stress-respons. Ik ontdekte dat roken verband houdt met hypercortisolemie en dat stoppen met roken de verhoogde cortisolafschijning omkeert. Hoofdstuk 2.2 presenteert determinanten van de negatieve terugvoerfunctie van de HPA-as die werd gemeten met behulp van een zeer lage dosis (0,25 mg) dexamethason suppressietest. In deze studie vond ik dat vrouw zijn, een laag inkomen, gebrek aan lichaamsbeweging, een instrumentele handicap en roken betrekking hadden op lage cortisol niveaus na dexamethason-inname. Klinisch relevante depressieve symptomen en angststoornissen waren echter gerelateerd aan een niet-suppressie van cortisol na dexamethason-inname. In hoofdstuk 2.3 onderzocht ik gemeenschappelijke varianten met betrekking tot plasma cortisol niveaus met behulp van een genoom-wijde associatie benadering. In deze studie vond en repliceerde ik gemeenschappelijke genetische varianten bij chromosoom 14q32 die SERPINA6 en SERPINA1 bevat. We hebben ook de functionele gevolgen van deze genetische varianten onderzocht en ontdekt dat zij het cortisolgehalte van plasma beïnvloeden via de cortisolbindende globuline (CBG). Ook lieten we een bewijs zien van de CBG-splitting die door de reactieve center lus werd geactiveerd. In hoofdstuk 2.4 beschrijf ik de resultaten van een genoom brede associatiestudies van het cortisol-niveau gemeten in speeksel die is afgenomen in de vroege ochtend. Verder beschrijf ik de SNP-gebaseerde erfelijkheid van speeksel- en plasmacortisol metingen. We hebben geen genoom-brede significante associatie gevonden tussen autosomale SNP's en cortisol speekselmetingen in de ochtend. Verder waren de SNP-gebaseerde schattingen voor de erfelijkheid van plasma en speekselcortisol zeer laag.

De vasculaire depressie hypothese is al lang bekend en is uitgebreid bestudeerd. Echter, deze studies zijn meestal klinisch met kleine steekproefgroottes. In hoofdstuk 3 heb ik deze hypothese herzien en de associaties van niet-klinische cerebrovasculaire veranderingen met depressie onderzocht. In hoofdstuk 3.1 vond ik dat zware herseninfarcten verband houden met het risico op depressie, terwijl subcorticale witte stof letsels verband houden met incidentele depressie bij niet-dementeerde ouderen. In hoofdstuk 3.2 heb ik verschillende

neuro imaging markers gebruikt om de associaties met depressie te testen. Ik ontdekte dat het volume van de laesie en de lacunair-infarcten in de witte stof verband houden met verschillende ernstgraden van depressie, terwijl cerebrale micro bloedingen alleen verband houden met ernstige depressie. In hoofdstuk 3.3 is de relatie tussen de cerebrale bloedstroom, vasomotorische reactiviteit en depressie longitudinaal geëvalueerd. Lage gemiddelde bloedsnelheid bij baseline werd longitudinaal geassocieerd met depressieve symptomen en depressieve stoornis. Ook verminderde vasomotorische reactiviteit was gerelateerd aan depressieve stoornissen bij mensen zonder dementie en zonder herseninfarcten. In hoofdstuk 3.4 hebben we de associatie van plasma amyloïde beta (Aβ) niveaus met depressieve syndromen in dementie-vrije mensen geëvalueerd. We vonden dat hoge niveaus van Aβ1-40 cross-sectioneel gerelateerd waren aan klinisch relevante depressieve symptomen en deze associatie werd verklaard door de prodromale fase van dementie. In longitudinale analyses bleken lage Aβ1-40- en Aβ1-42-niveaus verband te houden met een hoog risico op incidentele klinisch relevante depressieve symptomen bij mensen zonder prodromale fase van dementie tijdens de follow-up.

Hoofdstuk 4 rapporteert de resultaten van genoom-wijde associatie studies in depressie. In hoofdstuk 4.1 presenteert ik de resultaten van twee eerder gepubliceerde genoom-wijde associatie studies die afzonderlijk bij depressieve symptomen en stoornissen werden uitgevoerd. Ik vond en repliceerde een locus in het FHIT gen dat verband houdt met de circadiane klok, oxidatieve stress en DNA-schade. In hoofdstuk 4.2 heb ik gemeenschappelijke genetische varianten in sub domeinen van de CES-D-schaal beoordeeld die bestaat uit: negatieve effecten, positieve effecten, somatische symptomen en interpersoonlijke relaties. Eén variant van het in het hersenen tot expressie komende melatonine receptor (MTNR1A) gen was gerelateerd aan de somatische symptomen. Het is bekend dat melatonine een rol speelt bij depressie. Somatische symptomen van depressie bevatten slaap, eetlust en vermoeidheid, het is dus een zeer aannemelijk gen voor dit cluster van symptomen. Deze variant is echter niet gerepliceerd.

In hoofdstuk 5 heb ik de belangrijkste bevindingen van de studies besproken en methodologische kwesties en klinische implicaties samengevat.
Chapter 7

PhD portfolio

List of publications

About the author

Acknowledgements
### PHD PORTFOLIO

**PhD Student:** Nese Direk  
**Erasmus MC Department:** Epidemiology  
**PhD Period:** 2009-2013  
**Research School:** NIHES

#### TRAINING

Master courses in Health Sciences, Genetic Epidemiology (NIHES)

**Erasmus Summer Programme**

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<td>A First Encounter with Next-generation Sequencing Data</td>
<td>2012</td>
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**Skills Courses**

- English Language 2010 1.4
- Introduction to Medical Writing 2010 1.1
- Working with SPSS for Windows 2010 0.15
- Basic Course on R, Molecular Medicine, Erasmus MC 2010 1.4

**Other Courses**

- Mood Disorders in Later Life, 165th APA Congress 2012 0.2

**Seminars and Workshops**

- Seminars at the Department of Epidemiology 2009-2013 4.0
- Psychiatric Epidemiology Research Meetings 2009-2013 4.0
- Psychiatry Meetings at the Department of Psychiatry 2010-2013 2.0

**Conferences and presentations**

- 10th World Congress of Biological Psychiatry, Prague, poster presentation 2011 1.0
- 15th World Congress of Psychiatry, young investigator awardee, Argentina 2011 1.0
- 165th American Psychiatric Association Annual Meeting, Philadelphia, poster presentation 2012 1.0
- CHARGE Consortium Meeting, Iceland poster presentation 2012 1.0
- 21st the World Congress of Psychiatric Genetics, Boston, travel awardee, poster presentation 2013 1.0

**TEACHING**

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<td>Supervising undergraduate students within the Minor program</td>
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<td>Supervising Aniek de Rooij for the master’s thesis on vascular depression</td>
<td>2011</td>
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<td>Supervising Heidi Saavedra Perez for the master’s thesis on grief</td>
<td>2012</td>
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<td>Supervising Isabel Olmos Perez for the research project on suicidality</td>
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**OTHER**

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<td>Peer review for scientific journals</td>
<td>2010-2013</td>
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A COMPLETE LIST OF PUBLICATIONS

Publications presented in this thesis

Chapter 2


Chapter 3


Chapter 4


Other publications


Nese Direk was born on August 2nd, 1979, in Istanbul, Turkey. She studied medicine at Istanbul University School of Medicine. She obtained her medical doctor degree in 2002 and joined to the 5-years psychiatry residency program at Istanbul University Department of Psychiatry in 2003. After her graduation as a specialist of psychiatry, she has started to work at the Department of Epidemiology, Erasmus Medical Centre where she has worked on the current thesis. She has worked with the Rotterdam Study data under the supervision of Prof. Dr. Henning Tiemeier. During her time at the Erasmus Medical Centre, she obtained her the degree of Master of Science in Health Sciences (Genetic Epidemiology) in 2012. From 2013 to 2014, she worked as a post-doc researcher at the German Center for Neurodegenerative Disorders with Prof. Dr. Monique Breteler. She mainly focused on integrating the stress and psychiatric traits within the Rheinland Study. Then she moved to Izmir, Turkey in 2014. Since than she works as an assistant professor at the Department of Psychiatry, Dokuz Eylul University, Izmir. Her current work at the university focuses on vascular, genetic and hormonal markers of mood disorders.
ACKNOWLEDGEMENTS

When I started to this journey, I was not expecting that it will take such a long time. In general PhDs take long, especially if there are many conflicts and problems. But this was not the case for me. I loved my PhD period and being a student so much that probably I did my best to prolong it.

Henning, I think that it is impossible for me to just write few sentences about you here, I will try my best. I can easily say that you are one of the most important persons in my academic life. I do not consider you as a supervisor, only. Your enthusiasm, sarcasm, intelligence and honesty became the fuel of my passion in research. Thanks a lot for all the time that you have generously provided during supervisions, group meetings, coffee breaks (I mean your coffee breaks that I interrupted you very often to ask my ‘64 million dollar questions’) and Skype calls. After I left the Netherlands, my PhD process got very slow and from time to time seemed impossible to finish. One thing I will always remember is that how you kept encouraging me so that I could manage finishing it. I admire your patience. And also, many thanks for grinning and bearing with my B12 deficiency.

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Dear Olivera, it was great that I had chance to meet you and work together. But also, it is a pity that the time we had spent together was not long enough. Your sincerity and laughers were a good reason to be at work. You know, having someone knowing the taste of borek at the office is a cool thing. Also, having a friend who understands your concerns about the unrest in your country is also very helpful. It is a great pleasure for me to have a paranymph like you.

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this thesis. Professor Witte Hoogendijk, professor Fernando Rivadeneira, professor Roos van der Mast, I am very thankful that you have agreed to be opponents during my defence.

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Dear colleagues at the Dokuz Eylul University Department of Psychiatry, being a member of your group is an honour for me, I am very thankful that you have accepted me in your group. And dear professors and colleagues at the Department of Psychiatry, Istanbul University, it was a great pleasure to be your trainee and to work together. I have learned a lot from you all and I owe you so much.

I had asked. One day we will wander around the Soho streets together. Ez wek navê xwe bawerim.


And dear Gamze Ozcurumez, my non-Kleinian friend, thanks a lot for critical review of this text when I was in Berlin.

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