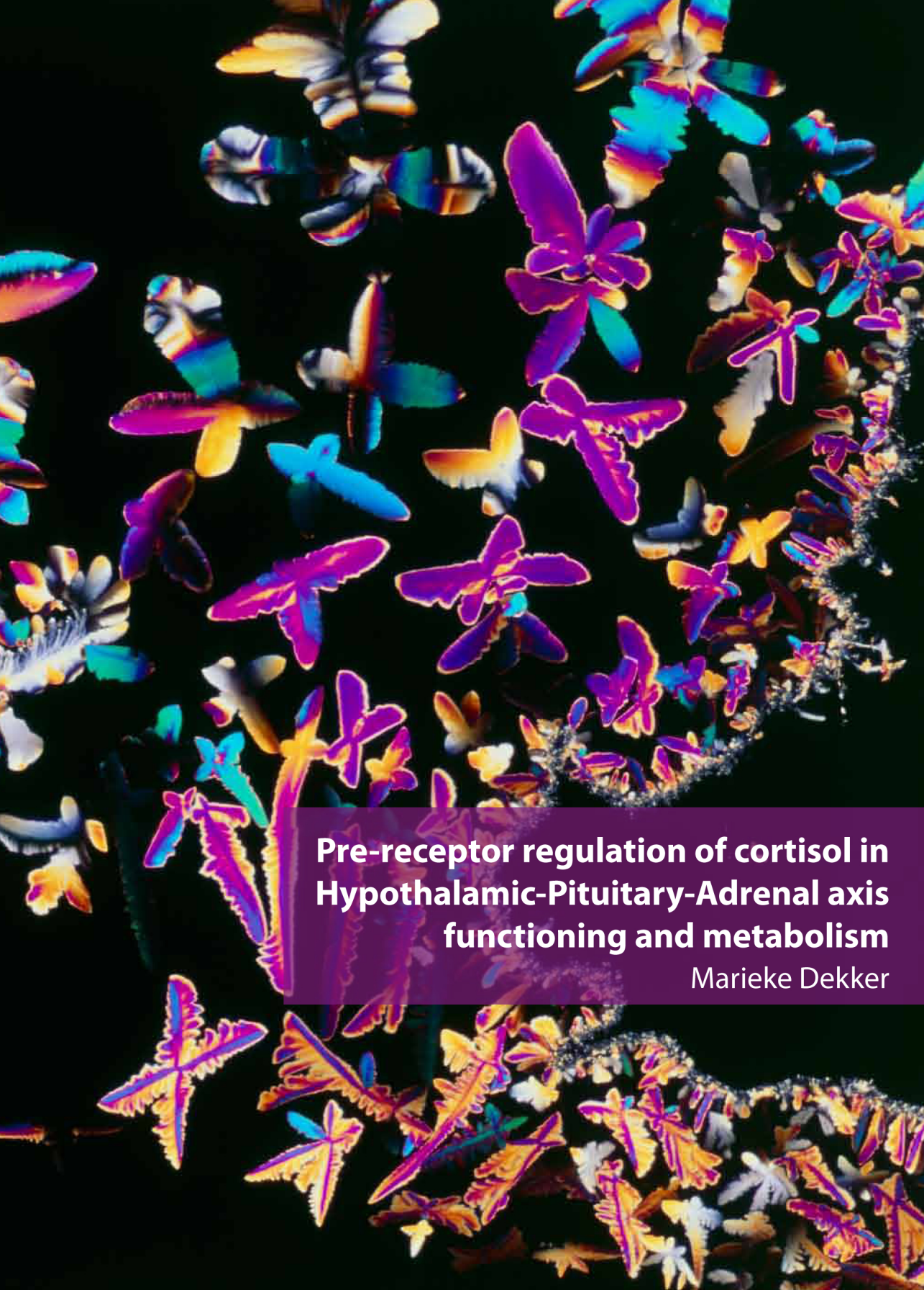


Pre-receptor regulation of cortisol in Hypothalamic-Pituitary-Adrenal axis functioning and metabolism

Marieke Dekker

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The research presented in this thesis was conducted at the Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands, in collaboration with the Department of Epidemiology of the Erasmus MC. The studies described in Chapter 3 were carried out at the Netherlands Institute for Neuroscience (NIN) and the Department of Endocrinology and Metabolism at the Academic Medical Center in Amsterdam. The study described in Chapter 7 was performed in collaboration with the Department of Neurology of the Erasmus MC.

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Chapter 1

General introduction

1.1 GLUCOCORTICOIDS

Glucocorticoids (GCs) are ubiquitous, nuclear hormones, which are essential for life[1]. In man, the main GC is cortisol, produced by the adrenals, endocrine glands that are situated on top of the kidneys[2]. Cortisol exerts its functions in nearly all tissues and is crucial in the mediation of the stress response[3]. Furthermore, cortisol is implicated in glucose and lipid metabolism, modulation of the immune system, maintenance of the vascular tone, bone formation, and skeletal and cardiac muscle function. Cortisol also affects brain function including memory modulation and mood regulation[4-10].

Because of its anti-inflammatory effects, cortisol and its synthetic analogues are now widely used in clinical practice. Indications include rheumatoid arthritis, inflammatory bowel disease, sarcoidosis, asthma, and dermatological diseases. Additionally, GCs have an important role in prevention of graft versus host disease in organ transplant patients and haematological malignancies[11-12]. Unfortunately, GC excess due to treatment or endogenous production, e.g. Cushing's syndrome, has serious adverse effects, such as truncal obesity, striae, osteoporosis, hypertension, hypokalaemia, hyperglycaemia, mood disorders and when prescribed to children growth retardation[13-14].

1.2 HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

The adrenal glands are composed of two functionally distinct parts: cortex and medulla. The medulla is part of the sympathetic nerve system and produces catecholamines. The cortex consists of three layers and produces steroid hormones. The outer zona glomerulosa is mainly under control of the renin-angiotensin system and secretes the mineralocorticoid aldosterone, whereas the secretion of cortisol and adrenal androgens from the zonae fasciculata and reticularis are centrally regulated by the negative feedback action of the hypothalamic-pituitary-adrenal (HPA) axis[15].

In response to diurnal cues and stressors, neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which induce corticotropin (ACTH) release from the anterior pituitary, a small endocrine gland at the base of the brain. ACTH in turn stimulates the release of GCs from the adrenal. Cortisol inhibits its own production at the level of the anterior pituitary and the PVN of the hypothalamus and thereby completes a negative feedback loop[2-3, 16] (Fig. 1).

In healthy persons, the HPA-axis shows a diurnal rhythm with peak cortisol levels approximately 30 to 45 minutes after awakening. This phenomenon is called the cortisol awakening response, abbreviated to 'CAR'[17]. During the day the cortisol levels decline gradually and its nadir is reached around 3.00 am. A few hours before awakening, cortisol starts to rise again. In response to various physical and psychosocial stressors (infection, trauma, fear, pain, social

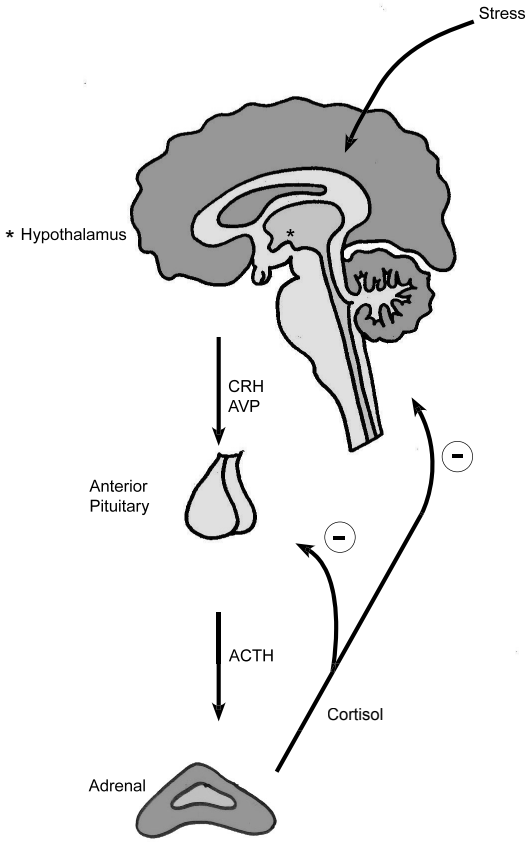


Figure 1. Schematic overview of the negative feedback loop of the HPA axis.

stressors, among others), the HPA-axis becomes activated and as a result the cortisol level increases[18-20]. In healthy persons, this stress response is once again terminated by means of the negative feedback loop of the HPA axis [21].

1.3 SENSITIVITY TO GCS, THE GLUCOCORTICOID RECEPTOR

In man, considerable interindividual variability but intraperson stability in sensitivity to GCs exists, suggesting a genetic influence on this sensitivity[22]. Sensitivity to GCs can be measured in vivo by the low-dose dexamethasone (Dex) suppression test and ex vivo by bioassays using mononuclear lymphocytes[23]. The effect of common genetic variation in the glucocorticoid receptor (GR) gene on these sensitivity tests has been studied extensively[24].

GCs exert their effects through binding to the GR. The GR is found in all cells throughout the body, and is essential for life. The GR is a member of the nuclear receptor family, which

includes 48 receptors, e.g., the estrogen, androgen, vitamin D, retinoic acid and the thyroid hormone receptor. Like the other nuclear receptors, the GR has a modular structure and has the following three functional domains: the amino-terminal transactivating domain (TAD), the highly conserved DNA binding domain (DBD), and the ligand binding domain (LBD)[25].

The GR gene (*NR3C1*) is located on chromosome 5q31, consists of 10 exons and has a gene size of approximately 180 kb[24]. Currently, more than 1600 single nucleotide polymorphisms (SNPs) have been identified in the GR gene region (db SNP database, build 132). A SNP is a substitution of a single nucleotide by another nucleotide. SNPs have a frequency of in average 1 per 100-1000 nucleotides[26]. A few SNPs in the GR have been associated with altered sensitivity to GCs, and might in part explain the large interindividual variation in treatment response and adverse effects to exogenous GCs, as well as interperson variability in sensitivity to endogenous GCs[27].

The ER22/23EK polymorphism (rs6189 and rs6190) consists of two linked SNPs and has been associated with a relative GC resistance. Carriers of this variant show decreased reduction of cortisol levels in the 1 mg dexamethasone (Dex) suppression test as well as reduced transactivation capacity in transfection experiments and bioassays using peripheral blood mononuclear lymphocytes of carriers[28-29]. In line with these findings, carriers of ER22/23EK SNP have a favourable metabolic profile, beneficial body composition, lower risk of dementia and white matter lesions, and this polymorphism is associated with better survival in elderly men[28, 30-32]. In contrast to the ER22/23EK, N363 (rs6195) and *Bcl*I (rs41423247) polymorphisms are associated with increased sensitivity to GCs in vivo. N363 carriers also showed higher transactivation capacity in bioassays testing the GR sensitivity[29, 33]. In concordance with these findings, both SNPs are related with an unhealthy metabolic profile and increased body weight[24].

In contrast to the aforementioned GR polymorphism, the GR-9β (rs6198) variant affects GR mediated transrepression but not transactivation[34], resulting in a decreased sensitivity to GCs. As the effects of the GR on inflammation are mainly mediated by transrepression, this polymorphism shows associations with outcomes related to the immune system: GR-9β is associated with Staphylococcus aureus carriage, rheumatoid arthritis, CRP and interleukin-6 levels, carotis intima media thickness, and myocardial infarction[35-38].

1.4 SENSITIVITY TO GCS, 11β-HSD1 AND 11β-HSD2

Sensitivity to circulating GCs at the tissue level is also regulated by the expression and activity of two enzymes: 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and Type 2 (11β-HSD2). The biological activity of cortisol depends on a β-hydroxyl group at C-11 of the steroid structure and interconversion to an 11-ketogroup results in an inactive metabolite, cortisone[39]. In vivo 11β-HSD1, converts inert cortisone to its active metabolite cortisol, whereas 11β-HSD2 converts cortisol to cortisone[40-44] (Fig. 2). Both enzymes are members of the short chain dehydrogenase

(SDR) superfamily. However, the 11β-HSD1 and 11β-HSD2 genes (*HSD11B1* and *HSD11B2*, respectively) are located on different chromosomes and only share 21% homology[45].

Only free cortisol is able to exert its function through binding to the glucocorticoid receptor (GR) in the target tissues. Ninety-five % of serum cortisol is bound to proteins like cortisol binding protein (CBG) and albumin. Although the adrenal glands may secrete small amounts of cortisone, the vast amount of circulating cortisone is derived from conversion of cortisol by 11β-HSD2 and its level is approximately one fifth of that of cortisol. However, because of a lower binding affinity to CBG of cortisone, free cortisone and cortisol levels are approximately the same[39].

11β-HSD1

HSD11B1 is located on chromosome 1q32-q41 and encodes a 34 kDa protein[45]. 11β-HSD1 is a member of the SDR superfamily. This is a very large family of enzymes, known to be NADPH or NAD dependent, with at least two domains: the cofactor-binding region and a centrally located active site, which binds the substrate[46]. 11β-HSD1 is situated in the membrane of the endoplasmatic reticulum (ER), and its active catalytic site is facing the lumen of the ER[41] (Fig. 2). The enzymatic activity is bi-directional, possessing both dehydrogenase (cortisol to cortisone), as well oxo-reductase (cortisone to cortisol) activities[47]. However, in intact cells or organs in vivo,

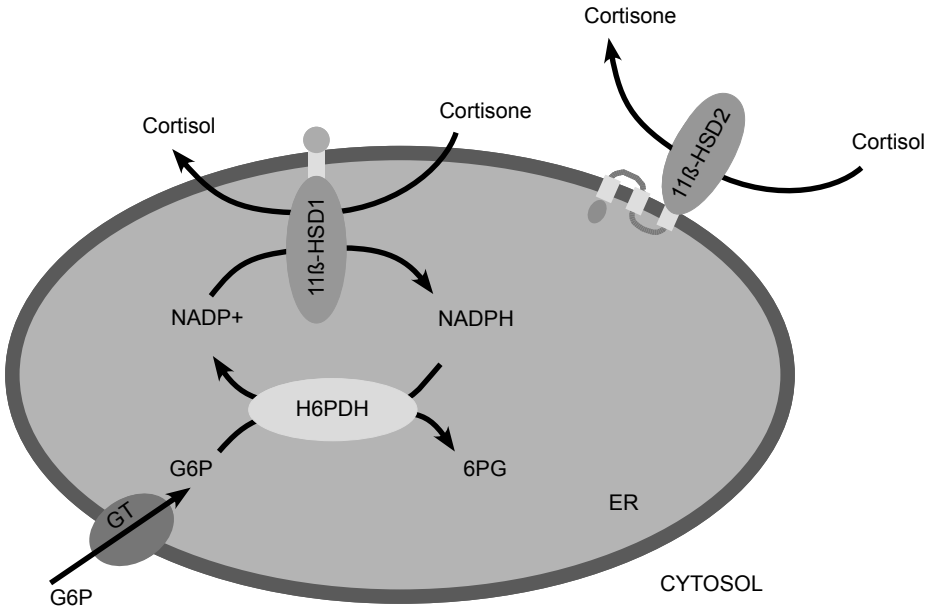


Figure 2. Schematic overview of interconversion of cortisone and cortisol by 11β-HSD1 and 11β-HSD2. H6PD generates NADPH by the conversion of glucose-6-phosphate (G6P) transported via the G6P translocase (GT), to 6-phosphogluconate (6PG) within the endoplasmatic reticulum (ER). 11β-HSD1 uses NADPH as cofactor allowing conversion of cortisone to cortisol[45].

11β-HSD1 acts predominantly as an oxo-reductase. The reaction direction is dependent on the availability of its co-factor NADPH[43]. The only source of NADPH in the ER is hexose-6-phosphate dehydrogenase (H6PDH). Mice with targeted disruption of the H6PDH gene (*H6PD*) are unable to convert 11-dehydrocorticosterone (11-DHC, the equivalent of cortisone in rodents) to corticosterone (the equivalent of cortisol in rodents). They show increased corticosterone to 11-DHC conversion, consistent with absence of 11β-HSD1 oxo-reductase activity, and a concomitant increase in dehydrogenase activity[42].

11β-HSD1 is widely expressed throughout the human body. It was first purified from the rat liver, but subsequent studies showed that 11β-HSD1 is also highly expressed in nearly all organs including adipose tissue, bone, skeletal muscle, skin tissue and the central nervous system[39, 48-49]. However, studies performed on human brain tissues are scarce. 11β-HSD1 expression is largely seen in cells expressing the GR, but not the MR, supporting the notion that 11β-HSD1 modulates access of GCs to the GR[44].

A rare clinical condition in humans in which a functional defect in the oxo-reductase function of 11β-HSD1 exist is cortisone reductase deficiency (CRD). This defect is illustrated by markedly elevated cortisone to cortisol metabolites, and an inability to convert exogenous cortisone to cortisol. Due to increased clearance of cortisol, the HPA axis gets activated, resulting in adrenal hyperplasia and symptoms of severe ACTH-mediated androgen excess like hirsutism, menstrual irregularities and acne[50-51].

11β-HSD2

HSD11B2 is located at chromosome 16q22 and encodes for a 44 kDa protein and is situated in the membrane of the ER. However, its cofactor-binding region and catalytic site are projected into the cytosol and 11β-HSD2 uses NAD instead of NADP as a cofactor (Fig. 2). It is a unidirectional dehydrogenase, converting cortisol to cortisone. 11β-HSD2 is mainly expressed in mineralocorticoid target tissues, like the kidney, colon and salivary glands, thereby preventing binding of cortisol to the MR[45, 52]. This protective mechanism is necessary because cortisol has the same binding affinity to the MR as aldosterone, but its serum concentration is 100 to 1000-fold higher than that of aldosterone[53].

Another important role of 11β-HSD2 is the protection of the developing fetus from the growth-inhibiting and pro-apoptotic effects of cortisol. It is highly expressed in the human placenta[54]. Perhaps as a further layer of defence, in rodents 11β-HSD2 is expressed in the developing brain. Central expression is observed from midgestation onward in a wide range of brain areas, but the expression is switched off as each nucleus develops[55-57]. Data of 11β-HSD2 expression in the developing human brain is lacking.

The syndrome of apparent mineralocorticoid excess (SAME) illustrates the importance and function of 11β-HSD2 in humans. Persons homozygous for deleterious mutation in *HSD11B2* have severe hypertension, hypokalaemia, suppressed plasma aldostesterone and renin levels. The profound hypokalaemia can cause rhabdomyolysis and nephrogenic diabetes insipidus[53,

[58-60]. Other symptoms include intra-uterine growth restriction, short stature and failure to thrive[45]. *HSD11B2* knockout mice show a 'SAME'-like phenotype[61-62]. Interestingly, those mice also exhibit neurodevelopmental abnormalities and a more anxious adult phenotype that are most likely the results of deleterious GC exposure during the critical stages of brain development[56, 63].

1.5 ROLE OF 11β-HSD1 IN HPA AXIS FUNCTIONING

Animal studies strongly suggest that 11β-HSD1 has a role in HPA axis regulation. 11β-HSD1 is widely expressed throughout the rodent brain, including negative feedback and regulatory sites of the HPA axis like the pituitary, PVN and the hippocampus[57, 64-66]. Harris *et al.* showed that 11β-HSD1 deficient mice have elevated plasma corticosterone and ACTH levels at the diurnal nadir, with a prolonged corticosterone peak. These mice also showed exaggerated ACTH and corticosterone responses to restraint stress, with a delayed fall of corticosterone levels after stress, suggesting impaired negative feedback of the HPA axis. Indeed, these mice showed a diminished capacity to suppress corticosterone levels after administration of exogenous GCs[67]. Interestingly, mice with targeted inactivation of *H6PD*, which generates NAPDH, the necessary cofactor for the reductase activity of 11β-HSD1, also exhibit impaired HPA axis negative feedback[68]. Recently, Carter *et al.*[69] discovered that HPA axis abnormalities in 11β-HSD1$^{-/-}$ mice are strain dependent. In contrast to 129/MF1 11β-HSD1$^{-/-}$ mice (study by Harris *et al.*[67]), 11β-HSD1$^{-/-}$ animals on a C57B1/6J background had normal basal plasma corticosterone and ACTH concentrations, and although these mice showed increased post stress corticosterone levels, they exhibited normal return to baseline corticosterone levels. In situ hybridization studies revealed that these mice have increased GR expression at central negative feedback sites, including the PVN and hippocampus, suggesting a compensatory mechanism to normalize HPA axis function.

Little is known about the role of 11β-HSD1 in human HPA axis regulation. Korbonits *et al.* detected both 11β-HSD1 mRNA as well as 11β-HSD1 immunoreactivity in the anterior pituitary. However, colocalization using immunofluorescence techniques revealed 11β-HSD1 expression only in the growth hormone- and prolactin-secreting cells, but not in ACTH-producing cells[70]. This suggests that 11β-HSD1 has no role in amplifying negative feedback at the level of the pituitary. Currently no data are available on human 11β-HSD1 expression at the PVN of the hypothalamus.

1.6 ROLE OF 11β-HSD1 IN THE METABOLIC SYNDROME

The metabolic syndrome (MetS) consists of a cluster of cardiovascular risk factors including insulin resistance, increased waist circumference, reduced high-density lipoprotein cholesterol (HDL) levels, elevated triglycerides (TG), and hypertension[71]. The common characteristics between MetS and hypercortisolemic states like Cushing's syndrome suggest that excessive exposure to GCs might be involved in the pathogenesis of the MetS[72-74]. However, although cortisol levels might be slightly elevated in the MetS, they are within the normal range and the clinical features cannot be explained by hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis alone[74-79].

In the late 1990's, Bujalska *et al.* found that human omental fat tissue had higher 11β-HSD1 activity than subcutaneous fat. The expression of this enzyme, and the differentiation of preadipocytes to adipocytes, was increased further after exposure to cortisol, suggesting that visceral adipose 11β-HSD1 expression could drive central obesity, one of the key features of the MetS. The possibility was raised that increased local generation of cortisol by 11β-HSD1 in omental fat tissue plays a pivotal role in the aetiology of MetS[72]. These ideas were further supported by the observation that transgenic mice overexpressing 11β-HSD1 in adipose tissue had increased levels of corticosterone and developed visceral obesity, as well as other features of the MetS, such as insulin resistant diabetes and hyperlipidemia[80]. 11β-HSD1 knockout mice appear more resistant to hyperglycaemia as a result of stress or obesity, which is consistent with the hypothesis that these mice have lower intracellular GC levels[62]. However, a similar weight gain when fed on a high-fat diet was observed for the 11β-HSD1 null mice, compared to the wildtypes[62, 81].

After the initial report of Bujalska *et al.* that 'central obesity may reflect Cushing's disease of the omentum' caused by overexpression of 11β-HSD1 in the omental fat, numerous other studies have explored the effects of 11β-HSD1 on obesity, insulin resistance, β-cell function, gluconeogenesis, lipid profiles and more[39, 82]. A detailed overview of all results is beyond the scope of this introduction, but has been covered in the excellent reviews by Tomlinson *et al.*[39] and Cooper *et al.*[82]. Overall, one can conclude that increased expression and activity of 11β-HSD1 is associated with an unfavourable metabolic profile.

11β-HSD1 expression seems to be important in compensatory mechanisms to acute weight loss. Obesity patients who were tested before and after substantial weight loss, showed a significant increase in 11β-HSD1 expression in adipocytes[83]. Likewise, the 11β-HSD1 expression decreased in adipose tissue of rodents when fed on a high-fat diet[84]. Moreover, studies suggest that 11β-HSD1 expression and activity in the liver is down-regulated in obesity[85]. This down-regulation, however, seems to be defective in patients who are insulin resistant[86].

Many authors proposed 11β-HSD1 inhibition as a novel drug target in the treatment of the MetS. Recently, the first clinical application of 11β-HSD1 inhibition has indeed become available. Rosenstock *et al*. found that when the 11β-HSD1 inhibitor INCB13739 was added to metformin monotherapy in type 2 diabetes patients exhibiting inadequate glycemic control,

significant reductions of fasting glucose and HbA1C levels were achieved. Moreover, this drug was well tolerated, body weight was decreased in the INCB13739 group, and in hyperlipidemic patients total cholesterol, low-density lipoprotein cholesterol (LDL) and TG were all significantly reduced[87].

1.7 ROLE OF HPA AXIS FUNCTIONING IN ATHEROSCLEROSIS

Atherosclerosis is a slowly progressive multistep process that starts with the formation of fatty streaks, which leads to the formation of atherosclerotic plaques. Although these plaques tend to be asymptomatic for decades, they may suddenly rupture, causing formation of a thrombus that will rapidly slow or stop the blood flow, leading to acute coronary syndromes (unstable angina or myocardial infarction), stroke or peripheral artery disease. A crucial event in the initiation of atherosclerosis is accumulation of LDL particles in the intima of the artery. Oxidized LDL particles trigger local inflammatory responses and the recruitment of mononuclear macrophages from the bloodstream. The uptake of atherogenic lipoproteins by these macrophages leads to lipid-laden foam cells and fatty streak formation, the initial lesion that may lead to atherosclerotic plaque formation. Established risk factors for atherosclerosis are tobacco smoking, impaired glucose tolerance, hypertension, high serum LDL and low serum HDL[88]. Albeit to a lesser extent than the traditional risk factors, chronic exposure to stressors and elevated cortisol levels may also be implicated in the pathogenesis of atherosclerosis[89-94].

By what mechanism would elevated cortisol levels promote the formation of atherosclerotic lesions? First, HPA axis dysregulation is associated with cardiovascular risk factors clustering in the metabolic syndrome, such as truncal obesity, dyslipidemia, high blood pressure and impaired glucose tolerance[74]. Next, Kanel *et al*. showed that morning serum cortisol was associated with a prothrombotic state in women with stable coronary disease, as reflected by higher von Willebrand, fibrinogen and CRP levels[95]. Furthermore, cortisol might directly affect vascular endothelial function, by reducing intracellular cyclic AMP (cAMP) and endothelial NO availability[96-97]. However, the exact role of cortisol on endothelial function has not yet been completely elucidated.

Several observations confirm the notion that cortisol is important in the aetiology of atherosclerosis. In patients with Cushing's syndrome, the mortality rate is four times higher than expected, with cardiovascular disease as the most frequent cause of death[98]. Ultrasound evaluation of the carotid arteries in this patient group revealed increased intima media thickness and frequency of atherosclerotic plaques[96, 99-101]. These findings were confirmed in studies with healthy volunteers and cardiovascular patient groups that showed that increased reactivity of the HPA axis, as measured by morning cortisol levels and the CAR, was associated with higher intima media thickness and coronary atherosclerosis[102-105]. However, other studies could not confirm these findings[106-107].

Only one large epidemiological study has evaluated the relation between cortisol levels and atherosclerotic plaque formation. Matthews *et al.* showed that flatter cortisol slopes, but not average cortisol levels, correlate with the presence of any coronary calcification in young and middle-aged adults[106]. Although this is an important finding, the prevalence of atherosclerosis was very low in this relatively young age group and other large studies in different age groups are needed to confirm these findings.

1.8 ROLE OF HPA AXIS FUNCTIONING IN DEPRESSION

Depression is characterized by a lasting depressed mood or loss of interest and pleasure, and a cluster of symptoms, such as sleep disturbances, loss of appetite or overeating, negative rumination, fatigue and poor concentration, suicidal ideation, and also apparent abnormalities of the HPA axis[108-109]. Patients with depression often have basal hypercortisolemia, as measured by increased 24-hour free urinary cortisol and elevated plasma and cerebrospinal fluid cortisol[110-112]. Moreover, many patients demonstrate impaired negative feedback, as shown by non-suppression of cortisol secretion following administration of exogenous GC and lack of inhibition of ACTH responses in the combined dexamethasone suppression/ corticotrophin-releasing hormone stimulation (DEX-CRH) test[113-115]. Interestingly, successful antidepressant treatment is typically accompanied by normalization of the HPA axis, while persistent HPA changes are a risk factor for relapse[116-120].

It is suggested that impaired negative feedback of the HPA axis in depression is the result of decreased sensitivity to GCs. In vitro and in vivo studies show that GR function is impaired in depression[121-123]. Furthermore, post-mortem studies have found reduced GR expression in the brains of depressed patients[124-125]. However, the exact molecular mechanisms underlying GC resistance in depression are not completely understood. Some authors suggest a role for P-glycoprotein (MDR1), a steroid transporter at the blood brain barrier, which has a role in regulating GC access to the brain. It is tempting to speculate that increased MDR1 function in depressed patients leads to reduced GR mediated actions in the brain by reducing intracellular cortisol levels[115]. Interestingly, antidepressants inhibit MDR1 function[126-128]. This has been shown for tricyclic antidepressants, as well as newer antidepressants such as fluoxetine, sertraline, paroxetine, and citalopram[115]. Other possible pathways include induced GC resistance by pro-inflammatory cytokines like interleukin (IL) 1 and IL-6, and impaired phosphorylation of the GR by the cAMP/Protein Kinase A pathway[115, 129-130]. The diminished regeneration of cortisol by 11β-HSD1 could be one of the molecular mechanisms underlying GC resistance in depression as well. However, at present no studies are available that confirm this hypothesis.

Recent studies show that subtle DNA changes in the GR itself are susceptibility factors for depression. ER22/23EK, a polymorphism that results in decreased sensitivity to GCs, is associated with increased risk of major depressive disorder[131-133] but a faster response to antidepressant

treatment[131]. Surprisingly, *BclI*, which has an opposite effect on GC sensitivity, has also been related to increased susceptibility to depression[131, 134-135]. Yet, this SNP was associated with a reduced response rate to antidepressants[136].

The role of pre-receptor regulation of cortisol by 11β-HSD1 in the aetiology of depression is largely unknown. Some authors evaluated the ratio of urinary cortisol and cortisone metabolites as a marker for overall 11β-HSD1 and 11β-HSD2 functioning in depressed patients. The results of these studies are conflicting: Raven *et al.* found increased cortisone to cortisol metabolite ratios in depressed women[137], whereas Poór found decreased cortisone to cortisol ratios in depressed men[138]. Another study that evaluated plasma cortisone to cortisol ratios, found no difference of overall 11β-HSD function between patients with a depressive disorder and controls at all[139]. Possibly, gender differences might explain the conflicting results.

Besides these few reports evaluating cortisone to cortisol ratios, other studies evaluating the role of 11β-HSD1 in depression are lacking. Given the fact that 11β-HSD1 is important in HPA axis regulation in animal models, *HSD11B1* is an interesting candidate gene for genetic association studies in search of susceptibility genes for depression.

1.9 METHODOLOGICAL ISSUES, *TAGGING SNP APPROACH*

One of the research aims of this thesis is to study the effects of common genetic variation in *HSD11B1* on hormonal measures, metabolic parameters and stress-mediated diseases. Using linkage disequilibrium (LD), it is possible to identify genetic variation in a chromosomal region without genotyping every SNP. Genetic variants, which are close together, tend to be inherited together, and are frequently (highly) correlated. This correlation is called 'LD'. Two different measures for the amount of LD exist: D' reflects the amount of recombination between two variants and r^2 is a measure for the correlation between two markers. In other words, how well does one polymorphism predict the presence of another polymorphism? Knowledge of common genetic variation and LD patterns has become available through the International HapMap Project. Using the HapMap data one can select a limited number of so-called 'tagging' SNPs to predict all known common genetic variation in the region of interest[26, 140]. In this thesis (Chapter 3 and Chapter 4) we used a tagging SNP approach to study the effects of genetic variation in *HSD11B1*.

1.10 METHODOLOGICAL ISSUES, *SALIVARY CORTISOL*

The assessment of salivary cortisol has become a valuable alternative to serum cortisol measurements. It is now widely used in research settings and more recently measurement of midnight salivary cortisol has proven valuable in the diagnosis of Cushing's syndrome[141-142].

In two research projects described in this thesis (Chapter 3 and Chapter 5) we used salivary cortisol measurements to determine diurnal cortisol patterns and cortisol levels at different time points throughout the day.

Salivary cortisol measurement has several advantages over serum cortisol measurement. The non-invasive sampling procedure is stress-free, reducing anticipatory stress and measurement-induced cortisol releases. Due to the laboratory independence of sampling, salivary cortisol can be measured at almost unlimited frequency and permits monitoring of diurnal cortisol patterns in the subjects' natural environment[142]. Furthermore, salivary cortisol reflects the unbound and thus biologically active fraction of cortisol[143]. There is an excellent linear correlation between salivary cortisol and free serum cortisol concentration. This relation is independent of changes in CBG concentration, and is thus the same for normal men and women, pregnant women and users of exogenous hormones[144]. However, salivary cortisol levels correspond to 70% of serum free cortisol due to conversion of cortisol to cortisone by 11β-HSD2 in the salivary gland[145].

1.11 AIMS OF THIS THESIS

The general aims of this thesis are to evaluate the effects of free cortisol, as measured by salivary cortisol, and the effects of pre-receptor regulation of cortisol by 11β-HSD1 on metabolic parameters, stress mediated diseases and HPA axis regulation.

Chapter 2. We studied the effects of two candidate SNPs in *HSD11B1* (83,557insA) and *H6PD* (R453Q) on body composition, blood pressure, glucose metabolism and adrenal androgens. In view of the known associations between increased cortisol levels and cognitive impairment, we also evaluated if these polymorphisms influenced susceptibility to dementia.

Chapter 3. We evaluated the 11β-HSD1 distribution in the human hypothalamus and the anterior pituitary gland, i.e. the two main negative feedback sites of the HPA axis by using immunocytochemistry. Colocalization of 11β-HSD1 with endocrine markers of the anterior pituitary and PVN was studied using fluorescence immunocytochemistry.

Chapter 4. The effect of common genetic variation in *HSD11B1* on HPA axis function was assessed using a tagging SNP approach. Salivary levels of cortisol and plasma androstenedione levels (an adrenal androgen) were used as measures of HPA axis activity. SNPs that showed consistent associations with salivary cortisol as well as androstenedione levels were also related to incident depression, because evidence suggests that dysregulation of the HPA axis is implicated in depression.

Chapter 5. The effect of common genetic variation in 11β-HSD1 on (features of the) MetS was investigated using a tagging SNP approach in two separate Caucasian populations.

Chapter 6. We studied the effects of salivary cortisol levels on atherosclerotic plaques of the carotid arteries. Two different aspects of HPA axis dynamics were evaluated: total cortisol exposure while awake and diurnal cortisol decline.

Chapter 7. In this additional study, we evaluated the effects of functional SNPs in the GR on susceptibility and clinical characteristics of Guillain-Barré syndrome (GBS). GBS is a post-infectious autoimmune-mediated polyneuroradiculopathy. *Campylobacter jejuni* is the most frequent antecedent infection in GBS. The far majority of those infections is not followed by GBS, indicating that genetic host factors may influence the development of such an immune response. The GR is an interesting candidate gene, because previously SNPs in the GR have been related to both susceptibility to develop autoimmune disease and microbial colonization.

Chapter 8. The thesis ends with a general discussion and future perspectives.

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Chapter 2

Lack of association of the 11β-hydroxysteroid dehydrogenase type 1 gene 83,557insA and hexose-6-phosphate dehydrogenase gene R453Q polymorphisms with body composition, adrenal androgen production, blood pressure, glucose metabolism, and dementia

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ABSTRACT

Context: Recently, it was proposed that a combination of the 83,557insA polymorphism in the 11ß-hydroxysteroid dehydrogenase type 1 gene (*HSD11B1*) and the R453Q polymorphism in the hexose-6-phosphate dehydrogenase gene (*H6PD*) interacts to cause cortisone reductase deficiency (CRD) when at least three alleles are affected.

Objective: The aim was to study the separate and combined effects of these polymorphisms on body composition, adrenal androgen production, blood pressure, glucose metabolism, and the incidence of dementia in the healthy elderly population.

Design/Setting/Participants: The Rotterdam Study (n = 6105) and the Frail Old Men Study (n = 347) are population-based cohort studies in the elderly.

Main outcome measures: Genotype distributions and influences of (combined) genotypes on body mass index, adrenal androgen production, waist-to-hip ratio, systolic and diastolic blood pressure, fasting glucose levels, glucose tolerance test, and incidence of dementia were measured.

Results: No influence of the *HSD11B1* 83,557insA (allele frequencies 22.0 and 21.5%) and *H6PD* R453Q (allele frequencies 22.9 and 20.2%) variants was found for the different outcome measures that were investigated, either separately or when at least three alleles were affected.

Conclusions: Two population-based studies among Caucasian elderly showed no evidence for (combined) effects of two polymorphisms in the *HSD11B1* and *H6PD* genes on body composition, adrenal androgen production, blood pressure, glucose metabolism, and incidence of dementia. Moreover, the high frequencies observed for these two polymorphisms do not correspond to the low incidence of CRD observed in the general population. Altogether, it is unlikely that these polymorphisms cause CRD.

INTRODUCTION

The enzymatic activity of 11ß-hydroxysteroid dehydrogenase type 1 (11β-HSD1), encoded by *HSD11B1*, is bidirectional with both dehydrogenase (cortisol to cortisone) and oxo-reductase (cortisone to cortisol) components[1-2]. *In vivo*, it acts predominantly as an oxo-reductase requiring nicotinamide adenine dinucleotide phosphate (reduced) for which in the liver hexose-six-phosphate dehydrogenase (H6PDH) has been shown to be the only source[3-6]. In other tissues this may be different, and the direction of the 11β-HSD1 reaction may depend on expression levels of H6PDH[4].

Recently, Draper *et al.*[5] concluded from a study in kindreds with cortisone reductase deficiency (CRD) that a combination of mutations in *HSD11B1* and *H6PD* interacts to cause CRD. They proposed a digenic triallelic mode of inheritance, in which three alleles, from two (or more) loci are necessary for trait manifestation. We studied the role of *HSD11B1* 83,557insA and *H6PD* R453Q, either separate or combined, on body composition, adrenal androgen levels, blood pressure, glucose levels and incidence of dementia in the elderly.

METHODS

Study groups

We genotyped a total of 6105 subjects for *HSD11B1* 83,557insA and *H6PD* R453Q from the Rotterdam Study, a population-based, prospective cohort study of subjects aged 55 and older[7]. In the Frail Old Men (FOM) Study, a population-based cohort study in 403 independently living men age 70 yr and older[8], we genotyped 347 Caucasians. Both studies were approved by the Medical Ethics Committee of the Erasmus MC and written informed consent was obtained from all participants.

DNA analysis

The appropriate Assay-by-Design mixes were designed, synthesized, and supplied by Applied Biosystems (Foster City, CA) (Table 1). Five-µl PCRs containing approximately 10 ng of DNA, 0.0625 µl 80x Assay-by-Design mix, 2.4375 µl water, and 2.5 µl Universal Master Mix (Applied Biosystems) were performed in 384-well plates. The reaction conditions were: 2 min 50 C, 10 min 95 C, followed by 40 cycles of 15 sec 92 C, and 60 sec 60 C. Plates were analyzed using the Applied Biosystems 7900HT Sequence Detection System and SDS version 2.0 software (Applied Biosystems).

Anthropometric measurements

Body weight and height were measured, and body mass index (BMI) was defined as weight divided by the square of height (kilograms/meters^2). The waist (umbilicus) and hip

Table 1. Sequences of Primers and Probes Used for Genotyping.

Gene	Primer/ probe name	Sequence 5′-3′
HSD11B1	Forward primer	CTT-ACC-TCC-TCC-TCT-GAA-CTT-TGC
	Reverse primer	TCC-TCC-TGC-AAG-AGA-TGG-CTA-TAT-T
	Wildtype-specific probe (FAM)	CAC-CAA-GAG-CTT-TT
	InsA-specific probe (VIC)	CAC-CAA-AGA-GCT-TTT
H6PD	Forward primer	TCT-GTC-CGA-TTA-CTA-CGC-CTA-CA
	Reverse primer	GGC-CAT-GGA-AGA-TAT-GGG-ATA-AGA-G
	453R-specific probe (FAM)	CTG-TGC-GGG-AGC-G
	453Q-specific probe (VIC)	CCT-GTG-CAG-GAG-CG

TaqMan primer and probe sequences used for *HSD11B1* 83,557insA and *H6PD* R453Q genotyping.

circumferences were measured, and the waist-to-hip ratio (WHR) was calculated. In the FOM Study, total lean body mass and fat mass were measured by dual energy x-ray absorptiometry as previously described[9-10].

Hormonal measurements

In a limited number of subjects of the Rotterdam Study, plasma levels of androstenedione (n = 1608) and dehydroepiandrosterone sulfate (DHEAS) (n = 1654) were determined. Plasma levels of androstenedione and DHEAS were estimated in 12 separate batches of samples using coated-tube RIAs purchased from Diagnostic Systems Laboratories, Inc. (Webster, TX). Due to the relatively small volumes of plasma available, all values reported are single-sample estimations. Intra-assay coefficients of variation, determined on the basis of duplicate results of internal quality control plasma pools with three different levels of each analyte, were less than 12% and 7% for androstenedione and DHEAS, respectively. Because inter-assay variations were relatively large (23% for androstenedione and 24% for DHEAS), results of all batches were normalized by multiplying all concentrations within a batch with a factor, which made results for the internal quality control pools comparable. This reduced inter-assay variations to 9% for androstenedione and 10% for DHEAS and was considered justified because the patterns of the results of these pools and the mean results for male and female sera in one assay batch were very similar.

In the FOM Study, serum DHEA and DHEAS levels were determined by RIA (Diagnostics Products Corporation, Los Angeles, CA) in nearly all subjects (n = 346). The intra-assay and inter-assay coefficients of variation for these assays were 3.8% and 2.1% and 8.6% and 5.1%, respectively.

Blood pressure measurement

Blood pressure was measured at the right upper arm in sitting position with a random-zero sphygmomanometer. Persons using blood pressure-lowering drugs and persons without data on blood pressure-lowering drugs were excluded from the statistical analyses with regard to blood pressure.

Assessment of glucose metabolism

In the Rotterdam Study, non-fasting serum blood samples were collected at baseline. Participants without diabetes mellitus (DM) underwent also a non-fasting glucose tolerance test (85% of the total population)[11]. In the FOM Study, only fasting glucose levels were measured.

Subjects with DM and subjects without data on DM were excluded from the statistical analyses with regard to glucose metabolism. In the Rotterdam Study, DM was defined as the use of blood glucose-lowering medication or random serum glucose concentration of at least 11.1 mmol/liter, or both. In the FOM Study, DM was defined as the use of blood glucose-lowering medication only.

Diagnosis of dementia

Dementia screening and diagnosis in the Rotterdam Study followed a three-step protocol as previously described[12].

Combined genotype analysis

Because Draper *et al.*[5] proposed a digenic triallelic mode of inheritance for the manifestation of CRD, we compared carriers of at least three affected alleles with the rest of the study group. The combination of three or four affected alleles is referred to as 'CRD genotype'.

Statistical analysis

Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, IL). All data are presented as means ± SEM. Statistical analyses on body composition, DHEA(S), androstenedione, blood pressure, and glucose metabolism were carried out by using the General Linear Model procedure and linear regression (P_{trend}), and were corrected for age and sex. Analysis on DHEA(S) was also corrected for smoking. Analysis on body composition and DHEA(S) were stratified for sex. Statistical analysis on the incidence of dementia was performed using Cox proportional hazards models adjusted for age and sex.

RESULTS

HSD11B1 83,557insA

In the Rotterdam Study, 3704 (60.7%) persons were defined as wild type, 2110 (34.6%) as heterozygous carriers, and 291 (4.8%) as homozygous carriers (allele frequencies, 78.0% reference allele and 22.0% variant allele). In the FOM Study, 217 (62.5%) subjects were defined as wild type, 111 (32.0%) as heterozygous carriers, and 19 (5.5%) as homozygous carriers (allele frequencies, 78.5% reference allele and 21.5% variant allele). Both populations were in Hardy-Weinberg equilibrium. The baseline characteristics of the participants of both studies are given in Table 2.

Table 2. Baseline Characteristics of the Rotterdam Study (n = 6105) and the Frail Old Men Study (n = 347).

Characteristic	Rotterdam Study	Frail Old Men Study
Sex, No. (%)		
male	2472 (40.5)	347 (100)
female	3633 (59.5)	0 (0)
Age (yrs)	69.5 (9.1)	77.6 (3.4)
Current Smoking, No. (%)		
yes	1347 (22.1)	60 (17.3)
no	4585 (75.1)	286 (82.4)
missing	173 (2.8)	1 (0.3)
Diabetes Mellitus, No. (%)		
yes	614 (10.1)	-
no	5089 (83.4)	-
missing	402 (6.6)	-
Use of Glucose Lowering Medication, No. (%)		
yes	261 (4.3)	30 (8.6)
no	4137 (67.8)	316 (91.1)
missing	1707 (27.9)	1 (0.3)
Glucose Tolerance Test (mmol/l)	6.9 (3.1)	-
Fasting Glucose (mmol/l)	-	5.8 (2.7)
Use of Blood Pressure lowering medication, No. (%)		
yes	1979 (32.4)	138 (39.8)
no	4124 (67.6)	208 (59.98)
missing	2 (0.0)	1 (0.3)
Systolic Blood Pressure (mmHg)	139.3 (22.1)	155.8 (23.9)
Diastolic Blood Pressure (mmHg)	73.7 (11.4)	83.5 (11.0)
Weight (kg)		
Male	78.4 (10.7)	76.3(10.2)
Female	69.4 (11.3)	-
Height (cm)		
Male	174.7 (6.8)	172.9 (6.3)
Female	161.2 (6.6)	-
BMI* (kg/m^2)		
Male	25.7 (3.0)	25.5 (2.9)
Female	26.7 (4.0)	-
WHR‡		
Male	0.96 (0.07)	0.98 (0.05)
Female	0.87 (0.09)	-
Trunk fat mass (kg)	-	10.7 (2.5)
Trunk lean mass (kg)	-	25.6 (29.7)
Total lean mass (kg)	-	52.1 (5.4)
Total fat mass (kg)	-	21.1 (5.5)
DHEA† (nmol/l)	-	7.39 (3.91)
DHEAS# (µmol/l)		
Male	4.4 (2.9)	1.98 (1.39)
Female	2.6 (2.0)	-
Androstenedione (nmol/l)		
Male	4.4 (1.9)	-
Female	3.6 (1.9)	-

Data are presented as means (SD) unless stated otherwise. *BMI = body mass index. ‡WHR= waist-to-hip ratio. †DHEA = dehydroepiandrosterone, #DHEAS = dehydroepiandrosterone sulfate

In the Rotterdam Study, no associations with weight, BMI, waist circumference, hip circumference, or WHR were found. In female carriers, a trend towards higher length was found (wild types, 161.0 ± 0.14 cm; heterozygous carriers, 161.5 ± 0.19 cm; homozygous carriers 161.8 ± 0.51 cm; $P_{ANOVA} = 0.06$; $P_{trend} = 0.02$). There were no differences in adrenal androgen levels, blood pressure, glucose metabolism or incidence of dementia. In the FOM Study, no differences in weight, BMI, WHR, trunk fat mass, trunk lean mass, total lean mass, and total fat mass were found. However, there was an association with lower height in carriers (wild types, 173.4 ± 0.43 cm; heterozygous carriers, 172.2 ± 0.59 cm; homozygous carriers, 170.4 ± 1.44 cm; $P_{ANOVA} = 0.049$; $P_{trend} = 0.02$). There were no differences in adrenal androgen levels, blood pressure, or glucose metabolism.

H6PD R453Q

In the Rotterdam Study, 3655 (59.9%) subjects were defined as wild type, 2105 (34.5%) as heterozygous carriers, and 345 (5.7%) as homozygous carriers (allele frequencies 77.1% reference allele, 22.9% variant allele). In the FOM Study, we found 224 (64.6%) homozygous wild-type carriers, 106 (30.5%) heterozygous carriers, and 17 (4.9%) homozygous carriers (allele frequencies, 79.8% reference allele and 20.2% variant allele). Both populations were in Hardy-Weinberg equilibrium.

For the Rotterdam Study, we found no associations with weight, height, BMI, or waist circumference. Male carriers had a smaller hip circumference compared with wild types (wild types, 98.7 ± 0.17 cm; heterozygous carriers, 98.2 ± 0.23 cm; homozygous carriers, 97.2 ± 0.60 cm; $P_{ANOVA} = 0.02$; $P_{trend} = 0.005$). In female carriers, a significant trend towards higher WHR was found (wild types, 0.87 ± 0.002; heterozygous carriers, 0.87 ± 0.003; homozygous carriers, 0.88 ± 0.006; $P_{ANOVA} = 0.10$; $P_{trend} = 0.04$). No differences in adrenal androgen levels, blood pressure, glucose metabolism, or incidence of dementia were found. In the FOM Study, no statistically significant differences were found for the investigated outcome measures.

Combined genotype groups

In the Rotterdam Study, 233 persons (3.8%) presented with the ´CRD genotype´ *vs.* 14 persons (4.0%) in the FOM Study. In both the Rotterdam Study, and the FOM Study, no statistically significant differences were found for the body composition parameters or other investigated outcome measures, including androgen levels.

DISCUSSION

Recently, Draper *et al.*[5] proposed a triallelic mode of inheritance in which at least three distinct alleles from two (or more) loci (*HSD11B1* and *H6PD*) are necessary for trait manifestation of CRD. However, in a recent study by San Millan *et al.*[13], it was shown that triallelic genotypes

of *HSD11B1* 83,557insA and *H6PD* R453Q single nucleotide polymorphisms (SNPs) do not always cause CRD. However, they found that PCOS patients had higher allele frequencies of *H6PD* R453Q compared with controls and that PCOS patients homozygous for *H6PD* R453Q had increased cortisol and 17-hydroxyprogesterone levels.

White showed that the *HSD11B1* 83,557insA and *H6PD* R453Q SNPs occur more frequently than previously reported but found no differences for BMI, WHR, visceral adiposity, insulin sensitivity, testosterone, FSH or LH (females), the risk of PCOS, or an effect on urinary free cortisol/cortisone ratio or the corticosteroid metabolite ratios[14]. However, Gambineri *et al*.[15] found that *HSD11B1* 83,557insA was significantly related to PCOS status, lower 0800-0830 h plasma cortisol, and higher cortisol response to $ACTH_{1-24}$ in all women with PCOS, and with higher DHEAS levels, greater suppression of DHEAS by dexamethasone, and lower fasting plasma LDL-cholesterol levels in lean PCOS women.

In our study, we showed that *HSD11B1* 83,557insA and *H6PD* R453Q are relatively common in the elderly population. Because a considerable part in both of our elderly study groups (Rotterdam Study, 3.8%; FOM Study, 4.0%) is the carrier of at least three affected alleles, it is very unlikely that these SNPs interact to cause CRD. Moreover, we showed that carriers of at least three affected alleles do not have higher androgen production, which is the key factor causing the symptoms observed in patients with CRD.

In a recent study, Lavery *et al.*[6] showed that *H6PD* knockout mice have a profound switch in 11βHSD activity from oxo-reductase to dehydrogenase, increasing the corticosterone clearance resulting in a reduction in circulating corticosterone levels. This demonstrated a critical requirement of H6PDH for 11βHSD1 oxo-reductase activity. However, in our study, no reliable effect of *H6PD* R453Q was found.

We conclude from our study, in two independent elderly populations, that we have not been able to detect any influence of the *HSD11B1* 83,557insA and *H6PD* R453Q SNPs, either separately or when using 3 or more affected alleles on body composition, adrenal androgen production, blood pressure, glucose levels, or incidence of dementia in the elderly. We also demonstrated that the presence of at least three affected alleles is relatively common in those two populations. Taking this together, it is unlikely that these SNPs cause CRD. However, because Lavery *et al*[6] demonstrated the critical role H6PDH for 11βHSD1 oxo-reductase activity, it is important to search for other possible functional SNPs in the *HSD11B1* and *H6PD* genes.

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Chapter 3

11β-Hydroxysteroid dehydrogenase type 1 expression in the human anterior pituitary and hypothalamus

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ABSTRACT

Objective: 11β-Hydroxysteroid Dehydrogenase type 1 (11β-HSD1) converts the inert hormone cortisone to active cortisol and is implicated in hypothalamus-pituitary-adrenal (HPA) axis regulation and in the stress response in animal studies. The aim of the present study was to report the distribution of 11β-HSD1 in the human hypothalamus and the anterior pituitary gland, i.e., the main negative feedback sites for cortisol.

Methods: We used polyclonal antisera raised against 11β-HSD1 for immunocytochemistry in a systematic selection of consecutive sections of six hypothalami and pituitaries from human post-mortem autopsy samples. Fluorescence immunocytochemistry was used to study the colocalization of 11β-HSD1 with hormonal markers in the hypothalamic paraventricular nucleus (PVN) and the anterior pituitary.

Results: 11β-HSD1 was expressed in many nuclei of the hypothalamus, most prominently in the PVN and the supraoptic nucleus (SON). In the PVN, 11β-HSD1 appeared to colocalize with vasopressin- (AVP), oxytocin- (OXT) and corticotropin-releasing hormone- (CRH) immunoreactive neurons. In the anterior pituitary, 11β-HSD1 was expressed in the hormone producing cells as well as folliculostellate cells. We were unable to visualize an 11β-HSD1 signal using fluorescence immunocytochemistry in the pituitary.

Conclusion: 11β-HSD1 is expressed at the two main feedback sites for cortisol, i.e., the PVN and anterior pituitary. In the human PVN, 11β-HSD1 is expressed by CRH-, AVP- and OXT-producing neurons. Our observations suggest that hypothalamic production of cortisol by 11β-HSD1 may modulate human HPA axis activity.

INTRODUCTION

Glucocorticoids (GCs) have numerous effects throughout the human body, including the regulation of metabolic and homeostatic processes and mediation of the stress response. GC secretion is regulated by the hypothalamic-pituitary-adrenal (HPA) axis[1]. In response to diurnal cues and stressors, neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing hormone (CRH) and vasopressin (AVP), which induces corticotropin (ACTH) release from the anterior pituitary[2]. ACTH stimulates the release of GCs from the adrenal. In man, the main GC is cortisol[3]. Cortisol inhibits its own production by inhibiting ACTH release at the level of the anterior pituitary, as well as CRH at level of the PVN and completes thereby a negative feedback loop.

Local, tissue specific cortisol concentrations are regulated by the expression and activity of two enzymes, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2). In vivo, 11β-HSD1 converts cortisone to cortisol whereas 11β-HSD2 inactivates cortisol to its inert 11-ketoderivate, cortisone. 11β-HSD1 is widely expressed throughout the human body[7]. 11β-HSD2 expression, on the contrary, seems to be confined to aldosterone target tissues, like the distal nephron, colon, and salivary and sweat glands, where it prevents cortisol to bind to the otherwise non-selective mineralocorticoid receptor[8].

Animal studies suggest that 11β-HSD1 plays a role in hypothalamic-pituitary-adrenal axis (HPA axis) regulation. In rodents, significant 11β-HSD1 expression is observed at the central negative feedback sites of the HPA axis: the anterior pituitary, the PVN and the hippocampus[7, 9-10]. Furthermore, Harris *et al*. showed that 129/MF1 mice lacking 11β-HSD1 activity showed elevated basal plasma corticosterone and ACTH levels and exaggerated ACTH and corticosterone responses to restraint stress, suggesting diminished GC feedback[11]. Recently, Carter *et al.* discovered that these HPA axis abnormalities are strain dependent: 11β-HSD1$^{-/-}$ mice with a C57B1/6J background have increased glucocorticoid receptor expression in areas of the brain involved in negative feedback, i.e., the PVN and hippocampus, suggesting a compensatory mechanism to normalise feedback control. Indeed, these mice have normal basal plasma corticosterone and ACTH concentrations and although these mice show increased post-stress corticosterone levels, they exhibit normal return to baseline corticosterone levels[12].

Only few studies are available on the expression of 11β-HSD isoenzymes in the human pituitary. In post-mortem human anterior pituitary, Korbonits *et al.* showed positive 11β-HSD1 immunoreactivity in growth hormone and prolactin-secreting cells, whereas no colocalization was observed with ACTH-, thyroid-stimulating hormone-, or luteinizing hormone- producing cells[13]. No 11β-HSD2 immunoreactivity was detected. By contrast, in ACTH-secreting adenomas 11β-HSD2 immunostaining was strongly positive in seven out of nine ACTH-secreting pituitary tumors, suggesting a possible explanation for the re-setting of GC feedback control in Cushing's disease[13]. So far no reports are available on the 11β-HSD expression in the human hypothalamus.

Studies on the functional neuroanatomy of 11β-HSD1 are likely to provide us with more insight in its possible role in the human HPA axis. Since these data are largely unavailable at present, we set two aims for the present study. First, we wanted to uncover the distribution of 11β-HSD1 in the human anterior pituitary and hypothalamus using immunocytochemistry. Our second aim was to investigate possible colocalization of 11β-HSD1 in the PVN of the hypothalamus and the anterior pituitary using combined immunocytochemistry.

METHODS

Human Brain Tissue

Post-mortem hypothalamic (n = 6) and pituitary (n = 6) tissue samples were obtained from The Netherlands Brain Bank (NBB) at The Netherlands Institute for Neurosciences. Permission was obtained for brain autopsies and, specifically, for the use of the tissues and clinical data for research purposes. Clinicopathological data of the patients are summarized in Table 1. Careful examination of the clinical and NBB records was performed to determine clinical diagnoses, cause of death, time of death, post-mortem delay (PMD), and fixation time. Patients without longstanding neurological or psychiatric disease were selected. Hypothalami were obtained from subjects who had not been treated with corticosteroids during at least four weeks prior to death. Hypothalami and pituitaries were fixed in 4% formaldehyde, dehydrated, paraffin-embedded and serially sectioned at 6 µm. The hypothalamic tissue blocks started rostrally at the level of the lamina terminalis, while the caudal border was at the level of mamillary bodies. Sections were mounted on Superfrost Plus slides (Menzel Gläser, Germany) and dried for at least two nights at 37° C. Prior to staining, slides were deparaffinised in xylene and rehydrated through a graded ethanol series. Subsequently, sections were rinsed in distilled water and in Tris-buffered saline (TBS, 3x 10 min).

Antisera

Anti-human 11β-HSD1 antibodies were raised in sheep (The Binding Site, Birmingham, UK). The following hypothalamic markers were used: mouse monoclonal anti-oxytocin (OXT, A-I-28), mouse monoclonal anti-vasopressin (AVP, VP III-D-7) and rat monoclonal anti-CRH (PFU83). Characteristics and specificity of the antisera have been described earlier[14-17]

Immunocytochemical staining, distribution studies

Six patients were selected to map the distribution of 11β-HSD1 in the hypothalamus. Every 100th section was selected to get a good overview of 11β-HSD1 expression throughout the hypothalamus. Immunocytochemistry was performed using the avidin-biotinylated-complex (ABC) method and consisted of the following steps. First, sections were pre-incubated in 5% milk-TBS (Elk Milk powder, Campina, the Netherlands, 0.05M Tris, 0.15M NaCl, PH 7.6) for 1h at

Table 1. Clinicopathological data of patients.

Subject	Sex	Age (years)	PMD (hours)	Fix (days)	Area Studied	Cause of death, Clinical diagnoses
95102	M	53	10	31	Pituitary	Circulatory failure, unsuccessful resuscitation, cardiac tamponade, coma
96075	M	76	6	ND	Pituitary	Heart failure, pulmonary embolism, Alzheimer's disease
95101	F	73	6	31	Pituitary	Cardiac failure, type 2 diabetes, heart failure, angina pectoris
96082	M	74	8	ND	Pituitary	e.c.i, Parkinson's disease
96009	M	86	7	31	Pituitary	Bronchopneumonia, Alzheimer's disease, heart failure, hypothyroidism
96006	F	90	3	31	Pituitary	Influenza, Alzheimer's disease, cachexia
97156	F	77	2	47	Hypothalamus	Septic shock, icterus, metastasized pancreas carcinoma, sepsis
98024	F	49	16	31	Hypothalamus	Shock, probably cardiac failure, myelodysplastic syndrome, acute myeloid leukemia, type II diabetes
97066	M	55	4	27	Hypothalamus	Multi-organ failure, HIV, hepatosplenomegaly, Hodgkin lymphoma, cachexia
98072	M	79	17	31	Hypothalamus	Haemorrhage in the brain stem, generalized atherosclerosis, with moderate chronic renal failure, diverticulosis coli
94039	M	78	ND	88	Hypothalamus	Electromechanical dissociation during heart catheterisation, ischemic heart disease, recent myocardial infarction
99046	F	89	5	36	Hypothalamus	Probable acute myocardial infarction, decompensatio cordis, severe left-sided cardiac failure based on mitral valve insufficiency and coronary sclerosis

Subject no = subject number, PMD = post-mortem delay, Fix = fixation time, ND = not determined.

room temperature (RT). Subsequently, sections were incubated with the first antibody (diluted 1:2000 in Supermix-milk [Supermix=SUMI, 0.05M Tris, 0.15M NaCl, 0.25% gelatin[18], 0.5% Triton X-100 {Sigma, Zwijndrecht, the Netherlands}, 5% milk powder (Elk Campina), pH7.6]) for 1h at RT followed by incubation overnight at 4˚C. The slides were rinsed in TBS (2x10 min), and incubated for 1 h at RT with rabbit-anti-sheep (1:400) in SUMI. After rinsing in TBS (2x10 min) the sections were incubated with ABC (1:800 in SUMI, Vector Laboratories, USA) for 1h at RT and subsequently rinsed in TBS (2x10 min). Finally, sections were incubated in 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) in TBS containing 0.2% ammonium nickel sulfate (BDH, Brunschwig, Amsterdam, The Netherlands) and 0.01% H_2O_2 for approximately 20 min. The reaction was stopped in aqua dest. The sections were dehydrated in graded ethanol series, cleared in xylene and cover slipped using Entellan (Merck). 11β-HSD1 staining of the pituitary followed the same procedures except for the following steps: no pre-incubation with TBS-milk was performed and the anti-11β-HSD1 antibodies were diluted 1:500 in SUMI.

Immunofluorescent staining, colocalization studies

To study colocalization patterns of 11β-HSD1 with CRH, AVP and OXT producing cells of the PVN, the same hypothalami as in the distribution studies were used. Immunocytochemistry was

performed using immunofluorescence and consisted of the following steps. First, sections were pre-incubated in 5% milk-TBS for 1h at RT. Next, sections were incubated with first antibody (anti-11β-HSD1, 1:50 in SUMI-milk), combined with a PVN marker (mouse monoclonal anti-AVP 1:50 in SUMI, mouse monoclonal anti-OXT 1:100 in SUMI, and rat monoclonal anti-CRH 1:10000) for 1h at RT and subsequently overnight at 4˚C. The next day, the slides were rinsed in TBS-milk (2x10 min) and incubated for 1 h at RT with biotinylated rabbit-anti-sheep (1:400 in SUMI, for OXT and AVP slides) or biotinylated goat-anti-rat (1:400 in SUMI, for CRH slides), followed by rinsing in TBS (2x10 min). The sections were incubated with ABC for 1h at RT and rinsed with TBS (2x10 min). Subsequently, incubation with biotinylated tyramide (1:750 in TBS-0.01% H_2O_2) for 30 min at RT followed. Sections were rinsed again with TBS (2x10 min) and incubated with donkey-anti-sheep conjugated to Cy3 and Streptavidine-Cy2 (respectively 1:200 and 1:150 in SUMI, for CRH and 11β-HSD1 staining) or incubated with donkey-anti-mouse coupled to Cy2 and Streptavidine-Cy3 (respectively 1:50 and 1:500 in SUMI, for OXT, AVP and 11β-HSD1 staining). The sections were then rinsed in water and coverslipped with Mowiol. Sections were then photographed by a confocal laser scanning microscope (CLSM 410, Zeiss).

RESULTS

A schematic overview of 11β-HSD1 immunoreactivity in the hypothalamus is given in Fig. 1. Table 2 shows the different 11β-HSD1 staining intensities observed in the hypothalamic nuclei. Expression was most prominent in the SON and PVN and was observed in magnocellular as well as parvocellular neurons of the PVN (Fig. 2A and Fig. 2B). Additional, although less intense 11β-HSD1 immunostaining was also present in the nucleus basalis of Meynert (NBM); suprachiasmatic nucleus (SCN); infundibular nucleus (IFN); tuberal lateral nucleus (NTL) and tuberomammilary nucleus (TMN). There was marked interindividual variation in distribution of 11β-HSD1 expression throughout the hypothalamus. For example, subject #97066 did not show 11β-HSD1 expression in IFN, whereas subject #99046 did not express 11β-HSD1 in NTL. Interestingly, subject #98072 did not show any 11β-HSD1 staining of the hypothalamus in three repeated immunostainings.

Double immunostaining in the PVN showed co-expression of 11β-HSD1 with AVP and OXT in all five subjects who expressed 11β-HSD1 in the PVN, except for subject #97066 (Fig. 3). In this subject, 11β-HSD1 was present in AVP immunoreactive PVN cells, but not in OXT neurons. Nearly all AVP and OXT producing cells co-expressed 11β-HSD1. 11β-HSD1 colocalised with CRH in three out of five patients (Fig. 3). The percentage of double-staining CRH producing cells differed between these patients: #97156 and #98024 showed clear colocalization with 11β-HSD1 in the majority of CRH immunoreactive neurons, whereas in patient #99046 colocalisation occurred less frequently. Both subject #97066 and #94039 had a very weak CRH signal, so colocalization could not be adequately assessed.

In the anterior pituitary, inhomogeneous staining of the endocrine as well as folliculostellate cells was observed (Fig. 4). We were unable to visualise an 11β-HSD1 immunofluorescence signal in the pituitary, so we were unable to examine 11β-HSD1 co-expression in the various hormone secreting cells of the anterior pituitary.

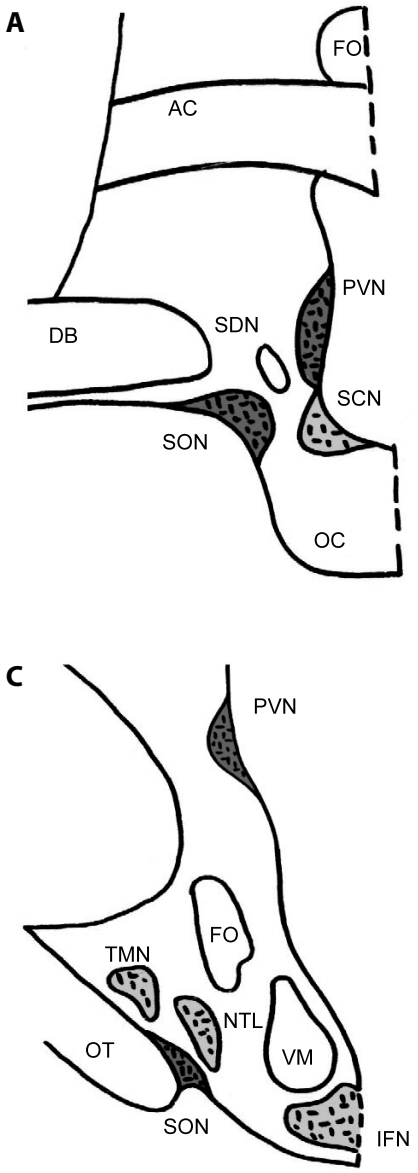


Figure 1. Schematic illustration of the distribution of 11β-HSD isoenzymes in the human hypothalamus.
Schematic illustration of 11β-HSD1 immunoreactivity in the human hypothalamus. Different shades of grey represent different staining intensity. AC = anterior commissure, DB = diagonal band of Broca, FO = fornix, IFN = infundibular nucleus, NTL = lateral tuberal nucleus, OC = optic chiasm, OT = optic tract, PVN = paraventricular nucleus, SCN = suprachiasmatic nucleus, SDN = sexually dimorphic nucleus, SON = supraoptic nucleus, TMN = tuberomammilary nucleus, VM = ventromedial nucleus.

Table 2. Staining intensities of 11β-HSD1 in the hypothalamus.

	97156*	98024*	97066*	94039*	99046*	98072*
PVN	+	++	+	+++	++	-
SON	+	++	+	+++	+++	-
NBM	+	++	+	++	++	-
SCN	+	+	+	+	++	-
IFN	-	++	-	++	++	-
NTL	+	+++	+	++	-	-
TMN	+	++	+	++	+	-

*numbers represent subject numbers. PVN = paraventricular nucleus, SON = supraoptic nucleus, NBM = nucleus basalis of Meynert, SCN = suprachiasmatic nucleus, IFN = infundibular nucleus, (NTL) = tuberal lateral nucleus, TMN = tuberomammilary nucleus.

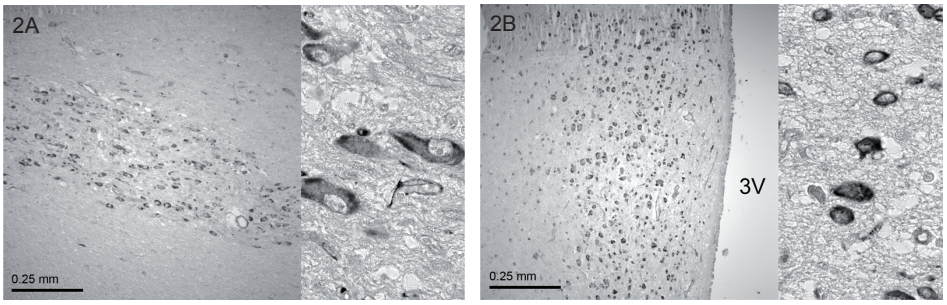


Figure. 2 11β-HSD1 immunostaining of SON and PVN.
A represents the supraoptic nucleus (SON).
B shows the paraventricular nucleus (PVN) 3V = third ventricle. Scale bars represent 250µm. Magnification 10x and 63x.

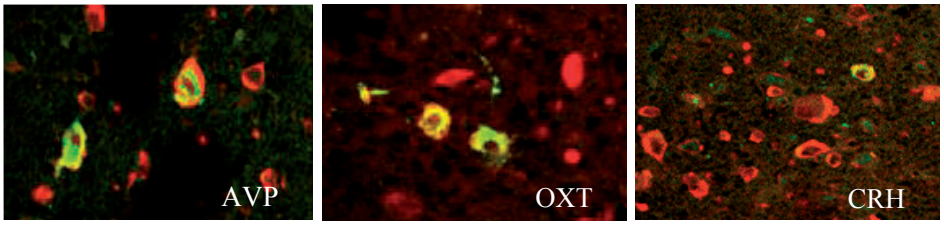


Figure 3. Colocalisation of 11β-HSD1 with hormone producing cells of the PVN.
Cells expressing 11β-HSD1 are stained red and cells expressing paraventricular nucleus (PVN) markers are stained green. When cells express 11β-HSD1 and a PVN marker, cells are stained yellow. 11β-HSD1 colocalises with vasopressin- (AVP), oxytocin- (OXT) and corticotropin-releasing hormone- (CRH) immunoreactive neurons in the PVN.

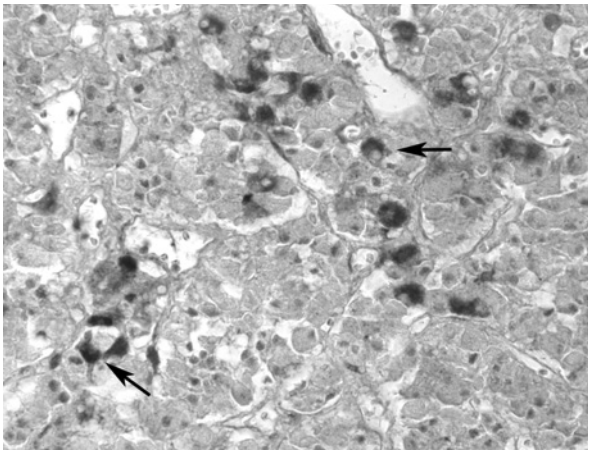


Figure. 4 11β-HSD1 immunostaining of anterior pituitary.
Arrows denote 1β-HSD1 positive endocrine cells. Magnification 63x.

DISCUSSION

In the present study we showed that 11β-HSD1 is expressed in the human brain in a number of hypothalamic nuclei, most prominently in the PVN and SON. Colocalization studies revealed that in the PVN, 11β-HSD1 was expressed in a large proportion of AVP-, and OXT-producing cells. The fraction of CRH immunoreactive cells in the PVN that co-expressed 11β-HSD1 showed marked interindividual variation and was generally less than for AVP and OXT. In line with the previous findings of Korbonits *et al.*[13], we found that 11β-HSD1 was expressed both in the hormone producing cells and the folliculostellate cells in the anterior pituitary. Unfortunately, we were unable to visualise 11β-HSD1 using immunofluorescence techniques, so we could not examine 11β-HSD1 colocalization in the anterior pituitary.

To our knowledge, this is the first study to report 11β-HSD1 expression in the human hypothalamus. Previous studies revealed that 11β-HSD1 is widely expressed throughout the rodent brain, including the cerebellum, many brain stem nuclei, (neo)cortex and hippocampus[7-8, 19]. In rodents, 11β-HSD1 was also expressed in the anterior pituitary and several regions of the hypothalamus including the PVN, SON, anterior medial preoptic area, lateral preoptic area and posterior arcuate nucleus. However, no expression in the SCN, periventricular nucleus and posterior hypothalamus was present[9-10, 20].

In our study, we found most prominent 11β-HSD1 immunoreactivity in the PVN and SON. These nuclei are both implicated in HPA axis regulation. The PVN lies adjacent to the third ventricle, from which it derives its name, and contains magnocellular as well as parvocellular cells. Axon terminals of the parvocellular cells of the PVN secrete, among other hypophysiotropic hormones, CRH and AVP into the long portal blood vessels of the zona externa of the median

eminence, which connects the hypothalamus with the anterior pituitary[18, 21]. In the anterior pituitary, CRH and AVP synergistically stimulate ACTH secretion from the corticotroph cells[6, 22-23].

The magnocellular neurons of the PVN and the SON are part of the hypothalamic-neurohypophysial system (HNS) and secrete two hormones: OXT and AVP. These magnocellular neurons project through the zona interna of the median eminence to the posterior pituitary, where upon appropriate stimulation OXT and AVP are secreted into the circulation[21, 24]. One of the main biological functions of the HNS is to maintain body fluid and electrolyte homeostasis. However, some authors suggest that the HNS is also implicated in modulating HPA axis activity influencing the HPA axis at least at three different levels[21, 25-26]. First, local release of OXT and AVP within the PVN and SON inhibits AVP and CRH secretion and thereby attenuates HPA activity[21, 27]. Second, some AVP and OXT are released in the hypophyseal portal system, where it modulates ACTH release[28-31]. Thirdly, OXT and AVP reach the anterior pituitary through the general circulation[32]. Most of these theories are based on animal research, so the exact role of the HNS on HPA axis functioning in man needs to be determined. However, some studies in depressed subjects confirm that OXT and AVP producing cells of the HNS may influence human HPA axis activity in man[18, 33-34].

In our study we found that 11β-HSD1 was expressed in the magnocellular as well as parvocellular cells of the PVN. Immunofluorescence techniques showed that 11β -HSD1 expression was present in the CRH, AVP and OXT producing cells. These findings are in line with previous animal studies in rats. Seckl *et al.* showed in rats that approximately 70% of magnocellular as well as 70% of parvocellular cells of the PVN contain 11β-HSD1[20]. Given the fact that the PVN is one of the main negative feedback sites of the HPA axis, one can hypothesize that 11β -HSD1 enhances intracellular cortisol concentrations within the PVN and thereby amplifies negative feedback, which in turn results in lower HPA axis activity.

This hypothesis is supported by animal studies as well as human data. Harris *et al*. showed that 129/MF1 mice lacking 11β-HSD1 activity showed elevated basal plasma corticosterone and ACTH levels and exaggerated ACTH and corticosterone responses to restraint stress, suggesting diminished GC feedback[11]. We previously showed that SNPs in 11β-HSD1 gene are associated with higher plasma cortisol and in postmenopausal women also with higher plasma androstenedione levels, suggesting that alterations in 11β-HSD1 also affects HPA axis function in humans (Chapter 4). However, these studies do not indicate at what level the HPA axis is influenced.

11β-HSD1 in other (hypothalamic) nuclei might also influence HPA axis function. As discussed previously, studies indicate that magnocellular AVP originating from the HNS might modulate HPA activity. Furthermore, SCN derived AVP plays a pivotal role in neuroendocrine and behavioural day/night rhythms, such as that of the HPA axis[35]. We can only speculate about the role of 11β-HSD1 in the other hypothalamic nuclei. Like elsewhere in the body, 11β-HSD1 may be assumed to be of importance in regulating access of cortisol to the GR, and subsequently have a role in cell metabolism, cell survival and apoptosis. Given the expression of 11β-HSD1 in the

human IFN, which is the human analogue of the rodent arcuate nucleus (Arc), hypothalamic 11β-HSD1 expression is likely to be implicated in appetite regulation of food intake[36]. Indeed, GCs are known to influence body weight and food intake[37], an effect that may be (partly) mediated through the hypothalamic pathways. Future studies are needed to evaluate the function of 11β-HSD1 in the various hypothalamic nuclei and in other brain areas.

Some methodological issues need to be addressed. The specificity of the anti-human 11β-HSD1 antibodies has been tested and reported based upon Western blot analysis using liver extracts and including antibodies preadsorbed with the immunizing peptide[14]. The specificity of this antibody should still be confirmed using brain tissues, like the anterior pituitary and hypothalamus. Next, the 11β-HSD1 antibody concentration needed in the colocalization study using fluorescent immunocytochemistry was relatively high, so background staining may have (partly) biased our results. For this reason, immunostainings with preadsorbed 11β-HSD1 antiserum will be performed to exclude this possibility. Studies using additional techniques like mRNA in situ hybridisation and qPCR will be needed to confirm our immunocytochemical results in the PVN. One patient did not express 11β-HSD1 in any of the nuclei of the hypothalamus. One possibility is that in this subject, 11β-HSD1 expression was below the detection limit of our immunostaining. A very low expression level in this patient may possibly be due to a long post-mortem delay of 17 hours.

In summary, we found that 11β-HSD1 is expressed in the anterior pituitary and very prominently in the human PVN and SON. Co-expression of 11β-HSD1 with CRH-, AVP- and OXT in the PVN, which is one of the main negative feedback sites of the HPA axis, suggests that 11β-HSD1 might modulate the human HPA axis. Future studies are needed to further elucidate the role of 11β-HSD1 in HPA axis functioning.

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Chapter 4

The effect of common genetic variations in 11β-hydroxysteroid dehydrogenase type 1 on hypothalamic-pituitary-adrenal axis activity and incident depression

Submitted for publication

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ABSTRACT

Background: Accumulating evidence suggests that hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is involved in depression. 11β-Hydroxysteroid Dehydrogenase type 1 (11β-HSD1) converts inert cortisone to active cortisol and is implicated in HPA axis regulation in animal studies. The aim of our study was to identify polymorphisms in 11β-HSD1 gene (*HSD11B1*) with consistent associations with increased HPA axis activity and relate those polymorphisms to depression.

Methods: Twelve SNPs, including eleven tagging SNPs, were selected using the HapMap database and genotyped in 4228 participants of the population-based Rotterdam Study. The main outcome measures were: salivary cortisol levels after awakening ($Cort_{aw}$), 30 minutes later ($Cort_{aw+30}$), at 1700h ($Cort_{1700h}$), at bedtime ($Cort_{bed}$) and plasma levels of androstenedione (in women only). SNPs that were significantly associated with cortisol as well as androstenedione levels were also related to incident depression.

Results: Rs11119328 was associated with higher $Cort_{bed}$ as well as higher androstenedione levels (p-value after correction for multiple testing: 0.01 and 0.04 respectively). Carriers of this polymorphism had an increased risk of an incident depression (Hazard Ratio [HR] 1.28, 95% Confidence Interval [CI] 1.03–1.59). Two other SNPs, which were in high linkage disequilibrium (LD) with rs11119328, were related to higher cortisol levels, but not with androstenedione levels.

Conclusions: We identified one SNP, which was associated with increased salivary cortisol levels at nadir as well as higher androstenedione levels. Moreover, this SNP was also associated with a higher risk of an incident depression. This suggests that 11β-HSD1 is implicated in human HPA axis regulation and susceptibility to depression.

INTRODUCTION

Glucocorticoids are centrally regulated by the negative feedback action of the HPA axis: cortisol inhibits pro-opiomelanocortin gene transcription in the anterior pituitary; and corticotrophin-releasing hormone gene transcription and peptide secretion in the hypothalamus[1]. Local, tissue specific cortisol concentrations are fine-tuned by two enzymes, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2). In vivo, 11β-HSD1 converts cortisone to cortisol whereas 11β-HSD2 converts cortisol to cortisone[2].

Animal studies strongly suggest that 11β-HSD1 also plays a role in central HPA axis regulation. In rodents, 11β-HSD1 expression is observed in the anterior pituitary, the PVN and the hippocampus[2-4]. Furthermore, 11β-HSD1-deficient-mice showed elevated basal corticosterone and ACTH levels and exaggerated ACTH and corticosterone responses to restraint stress, suggesting diminished glucocorticoid feedback[5].

In the present study we wanted to evaluate the associations of common genetic variation in *HSD11B1* with HPA axis activity using a tagging SNP approach. As markers for HPA axis activity we used salivary cortisol levels and in women also adrenal androgens. Our aim was to identify SNPs, which show consistent associations with increased HPA axis activity and relate those polymorphisms to incident depression. To test these hypotheses we used data from the Rotterdam Study, a population-based cohort study in an elderly population.

METHODS

Study Population

The present study was imbedded in the Rotterdam Study, a population-based cohort study of persons aged 55 and older[6]. A timeline of the different study surveys and measurements is given in Fig. 1.

Plasma Androstenedione Measurement

Analyses with androstenedione were performed in women only, because in postmenopausal women the adrenals are the main source of androgen production, and its release is under the control of the HPA axis. In a limited number of women who participated in the baseline examinations (1990-1993, n = 883) serum levels of androstenedione, which are stable in frozen plasma[7], were determined as described previously (See Chapter 2). Women using exogenous hormones (n = 39) were excluded. This resulted in a study population of 844 women.

Salivary Cortisol

During the fourth study survey (2002-2004) participants collected saliva samples at four different time points: directly after awakening ($Cort_{aw}$), 30 minutes later ($Cort_{aw+30}$), at 1700 h

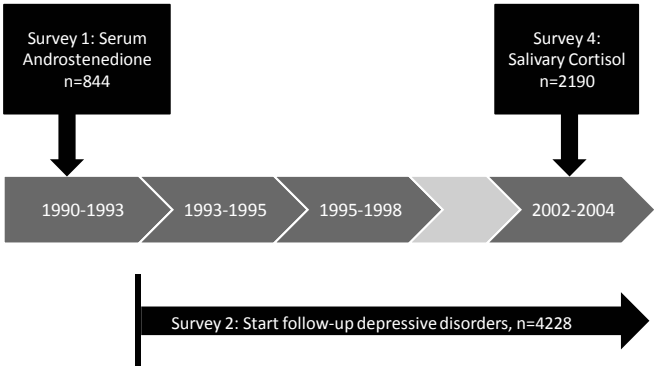


Figure 1. Timeline of different study surveys and measurements.

($Cort_{1700h}$) and at bedtime ($Cort_{bed}$). For each time point, cortisol values that were above the 98th percentile were excluded. This conservative cut-off was chosen due to the high number of outliers, in line with earlier studies[9]. Moreover, the 98th percentile of late-night salivary cortisol levels (13.4 nmol/L) corresponded approximately to the cut-off value used in the diagnosis of Cushing's syndrome. Persons using systemic glucocorticoids (n = 49) were excluded. This resulted in a study population of 2190 participants. See Chapter 6 for more details on saliva collection and cortisol measurements.

Incident Depression

Information on the occurrence of incident depressions during follow-up was obtained from (1) psychiatric examinations, (2) self-reported histories of depression, (3) medical records, and (4) registration of antidepressant use as has been described previously[10]. The psychiatric examination during visits consisted of a screening with the CES-D, and in the screen-positive participants a semi-structured interview by a trained clinician (Schedules for Clinical Assessment in Neuropsychiatry)[11]. The self-reported history of depression, solicited during the visits, included standardized questions to ascertain whether and when participants had suffered from a depressive episode, and if so whether they had been treated. Trained research-assistants scrutinized the GPs' medical records and copied the information about a potential depression. Two research physicians independently assessed this information according to a predefined protocol, and discussed discordant assessments.

An event was considered a depressive syndrome if one of the following conditions was met: a DSM-defined major depressive disorder or a minor depression diagnosed by a psychiatrist or another mental health professional, depressions recorded by a GP or other physician and a self-reported depression for which the participant consulted a GP or a mental health professional. We defined the date-of-onset as the day of the first report of symptoms, or the first prescription date of an antidepressant drug, whichever came first.

Table 1. Primers and probes.

SNP ID number	Primer / Probe	Sequence
rs17317033	Forward Primer	CAGGTCTTTGTATAAGATCATTATGCCTAGTT
	Backward Primer	TGACCTTTTACAAGGGTCTAGGTGTA
A > C	Wildtype-specific probe (VIC)	CACCTGTTGACTAAAT
	Minor allele (FAM)	ACCTGTGGACTAAAT
	Forward - Reverse	Reverse
rs846908	Forward Primer	GCAAGCAAAAGTGTCATTCAAAGAAC
	Backward Primer	GGAGAAACACCATTAGACAGATTTTGGA
G > A	Minor allele (VIC)	AATGTTCCACATTGGCT
	Wildtype-specific probe (FAM)	AATGTTCCACGTTGGCT
	Forward - Reverse	Forward
rs2235543	Forward Primer	TTCCTTCAGAGCCAGTGTTTCTC
	Backward Primer	CCCACCCTCCTTTCAGCTT
C > T	Wildtype-specific probe (VIC)	CCGACTCTGGTGAACT
	Minor allele (FAM)	CCGACTCTGATGAACT
	Forward - Reverse	Reverse
rs10082248	Forward Primer	CTCATTTCTATCCCCCAAATCAAAACC
	Backward Primer	CAATTTTGAGGCACTTGAGAAGCAT
G > A	Minor allele (VIC)	CTCTTTCAGATCTCC
	Wildtype-specific probe (FAM)	CTCTTTCGGATCTCC
	Forward - Reverse	Forward
rs4844880	Forward Primer	AGGGCATGGAGAGCATTTCAAT
	Backward Primer	GGGTGGAGGAATCATCTACTAACATTTATT
T > A	Minor allele (VIC)	TCAGGTACTATGAATAAA
	Wildtype-specific probe (FAM)	TCAGGTACTAAGAATAAA
	Forward - Reverse	Reverse
rs846910	Forward Primer	CTGTTGCTTGTGCTTGATTCCA
	Backward Primer	CAGCCAGGAATTCTCTCTGATTTGA
G > A	Minor allele (VIC)	TTTATTCTGGTGAGAATGA
	Wildtype-specific probe (FAM)	TTATTCTGGTGGGAATGA
	Forward - Reverse	Forward
rs3753519	Forward Primer	CTTGAAACAAATGCTAGCTGAAACAGT
	Backward Primer	CCCATCCCCCAAATCTTCTTTCTG
G > A	Minor allele (VIC)	CATTTTGTCTTTATTATTTTTTT
	Wildtype-specific probe (FAM)	ATTTTGTCTTTATTACTTTTT
	Forward - Reverse	Reverse
rs45487298	Forward primer	CTTACCTCCTCCTCTGAACTTTGC
	Reverse primer	TCCTCCTGCAAGAGATGGCTATATT
InsA	Wildtype-specific probe (FAM)	CACCAAGAGCTTTT
	InsA-specific probe (VIC)	CACCAAAGAGCTTTT
	Forward - Reverse	Forward
rs11119328	Forward Primer	CCCGTAATCATTGTTGCTTCATGTT
	Backward Primer	TGGTCTAGCAGGGATAAGTAACCT
C > A	Minor allele (VIC)	CTAAGTTCAGCCTAGCCTT
	Wildtype-specific probe (FAM)	TTCAGCCGAGCCTT
	Forward - Reverse	Reverse

Table 1. Primers and probes. (continued)

SNP ID number	Primer / Probe	Sequence
rs4844488	Forward Primer	GAGTTTGAGAAGGCTTACAACAAATACAT
	Backward Primer	GATCTATACTGAGTCTAATTTGGGCACAT
A > G	Wildtype-specific probe (VIC)	AACTAGGTCTTAAGAAAAG
	Minor allele (FAM)	ACTAGGTCTTAAGGAAAG
	Forward - Reverse	Forward
rs846906	Forward Primer	GTTTTACAACTGCTTGCCTCTGAAA
	Backward Primer	ACAATGTGCCTGCCTTTAAGGA
C > T	Minor allele (VIC)	AATCACTGTGAGTAAAAT
	Wildtype-specific probe (FAM)	CACTGTGGGTAAAAT
	Forward - Reverse	Reverse
rs11487867	Forward Primer	AAAACAGTATGGCAGTACCTCAGAAAA
	Backward Primer	GCCTCCTGCTTTCAATTCTTCTGT
G > T	Wildtype-specific probe (VIC)	ATTCTATTTCTGGGTATATAC
	Minor allele (FAM)	CATTCTATTTCTGGTTATATAC
	Forward - Reverse	Forward

DNA analysis

HSD11B1 tagging SNPs were selected using the HapMap database (Public Release #20). SNPs were selected to tag common variation (allele frequency cut-off 5%) 5kb upstream and 1kb downstream of *HSD11B1*. This resulted in the selection of 11 tagging SNPs. Since *HSD11B1* 83,557insA (rs45487298), which is in full LD with a T>G substitution at position 83,597 (rs12086634), has been extensively studied previously, we also included this polymorphism in our analyses.

The appropriate Assay-by-Design mixes were obtained from Applied Biosystems (Foster City, CA, USA). See Table 1 for primer sequences. Plates were analyzed using the Applied Biosystems 7900HT Sequence Detection System and SDS version 2.0 software (Applied Biosystems). To confirm the accuracy of genotyping results, 5% of the samples were re-genotyped. Percentage of concordance was higher than 99% for all SNPs.

Statistical Analyses

Hardy-Weinberg Equilibrium (HWE) was calculated using Chi-square analyses. Haploview (release 4.1) was used to determine the Linkage LD blocks and the r-squared values. All hormone levels were log transformed to normalise distributions.

First, linear regression models adjusted for age and sex were used to study the effects of *HSD11B1* SNPs on hormonal measures. P-values were corrected for multiple testing using a permutation procedure (10,000 permutations) available in the R package GenABEL version 1.6-4[12]. Next, we studied the effects *HSD11B1* SNPs on the incidence of depressive disorder using proportional hazard analyses adjusted for age and sex. To minimize multiple testing, only SNPs that were associated with higher salivary cortisol as well as higher androstenedione levels were included in these analyses. Subjects were analysed as carriers (1 or 2 copies of minor allele) versus non-carriers Statistical analyses were performed using SPSS for Windows,

release 15.0. Post-hoc analyses were performed to study the effect of *HSD11B1* SNPs on hormonal levels in persons without a history of depression.

RESULTS

Fig. 2 shows a schematic overview of *HSD11B1*, the selected SNPs and the observed minor allele frequencies. Additional information on the SNPs is provided in Table 2 and Fig. 3. Characteristics of the study population including means and standard deviations of the hormonal measures are given in Table 3.

Of the 12 SNPs, only one SNP, rs11119328, showed consistent associations with higher levels of salivary cortisol at bedtime as well as androstenedione in women (Table 4). These associations remained statistically significant after correction for multiple testing using permutation analyses. Two other SNPs, rs11487867 and rs45487298, which are in high LD with rs11119328 (Fig. 3), were significantly associated with higher salivary cortisol at bedtime, but not with androstenedione (Table 4). None of the analyses with other *HSD11B1* SNPs and hormonal measures reached statistical significance.

Post-hoc analyses showed that rs11119328 was also associated with higher $Cort_{bed}$ levels in persons without a history of depression at the fourth study survey (n = 1121, $B_{Cort(bed)}$ = 0,056 $Log_{nmol/L}$ (95% confidence interval [CI]: 0.015-0.097 $Log_{nmol/L}$), p = 0.007). A similar trend was not observed for androstenedione after exclusion of persons with a history of depression (B = 0.006 $Log_{nmol/L}$ (95% CI: -0,027-0,066 $Log_{nmol/L}$), p = 0.414, (n = 251).

We used Cox proportional hazard analyses to study the effect of *HSD11B1* SNPs on incidence of depression. For these analyses only one SNP, rs11119328, which was associated with higher

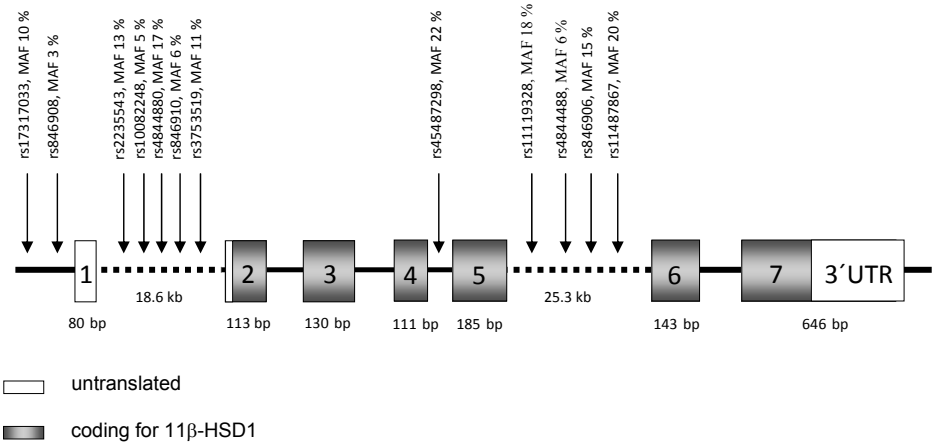


Figure 2. Schematic overview of *HSD11B1*, selected tagging SNPs and minor allele frequencies.

Table 2. Raw genotype frequencies, call rates, Hardy-Weinberg equilibria and minor allele frequencies of genotyped *HSD11B1* SNPS.

rs number	WT (n, %)	HZ (n, %)	HO (n, %)	Call rate, %	HWE	MAF, %
rs17317033	5172, 81.5	1123, 17.7	54, 0.9	96.7	0.42	9.7
rs846908	5849, 94.4	340, 5.5	5, 0.1	94.3	0.98	2.8
rs2235543	4779, 76.1	1393, 22.2	109, 1.7	95.6	0,52	12.8
rs10082248	5625, 90.3	587, 9.4	18, 0.3	94.8	0.52	5.0
rs4844880	4340, 68.9	1772, 28.1	185, 2.9	95.9	0.80	17.0
rs846910	5445, 88.6	690, 11.2	13, 0.2	93.6	0.07	5.8
rs3753519	4973, 80.0	1172, 18.8	74, 1.2	94.7	0.60	10.6
rs45487298	3892, 60.7	2219, 34.6	302, 4.7	97.6	0.53	22.0
rs11119328	4117, 67.8	1755, 28.9	203, 3.3	92.5	0.34	17.8
rs4844488	5655, 89.0	680, 10.7	18, 0.3	96.7	0.61	5.6
rs846906	4582, 72.0	1620, 25.4	165, 2.6	96.9	0.13	15.3
rs11487867	4015, 64.1	1995, 31.8	255, 4.1	95.4	0.72	20.0

Rs number = Reference SNP ID number, WT = wildtypes, HZ = heterozygotes, HO = homozygotes, HWE = Hardy-Weinberg Equilibrium, MAF = minor allele frequency.

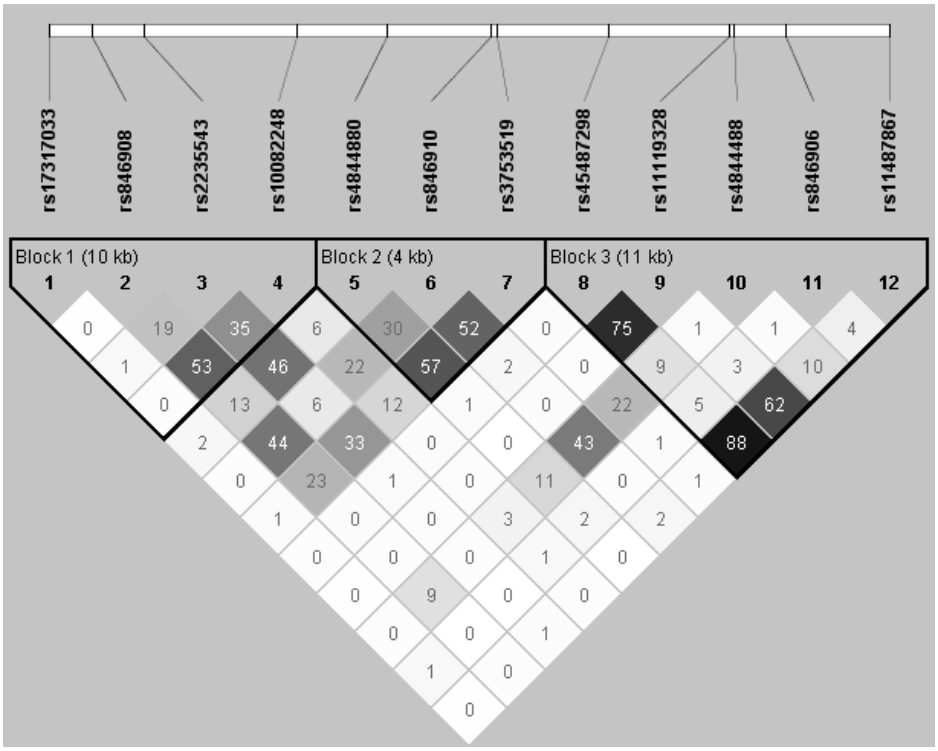


Figure 3. Linkage Disequilibrium plot of tagging SNPs in *HSD11B1*.
Numbers in picture denote the r-squared values between polymorphisms. All rs numbers represent tagging SNPs, except rs45487298, which represents an A-insertion.

Table 3. Characteristics of Study Population.

	Adrenal androgens* *Study Survey 1*, n = 844	Depressive Syndrome† *Study Survey 2*, n = 4228	Salivary Cortisol# *Study Survey 4*,n = 2190
Female gender, %	100	57.1	57.1
Age, yrs (SD)	71.7 (9.3)	69.8 (8.1)	75.0 (5.8)
BMI^, $kg*m^{-2}$ (SD)	26.7 (3.9)	26.5 (3.7)	27.4 (4.0)
Current Smoking, %	19.7	17.0	11.8
Androstenedione, nmol/L (SD)	3.60 (1.84)	NA	NA
$Cort_{aw}$‡, nmol/L (SD)	NA	NA	14.3 (7.78)
$Cort_{aw+30}$§, nmol/L (SD)	NA	NA	18.8 (9.41)
$Cort_{1700h}$‖, nmol/L (SD)	NA	NA	4.23 (2.97)
$Cort_{bed}$φ, nmol/L (SD)	NA	NA	2.24 (2.01)
Androstenedione, $Log_{nmol/L}$ (SD)	0.50 (0.22)	NA	NA
$Cort_{aw}$‡, $Log_{nmol/L}$ (SD)	NA	NA	1.08 (0.31)
$Cort_{aw+30}$§, $Log_{nmol/L}$ (SD)	NA	NA	1.18 (0.30)
$Cort_{1700h}$‖, $Log_{nmol/L}$ (SD)	NA	NA	0.52 (0.33)
$Cort_{bed}$φ, $Log_{nmol/L}$ (SD)	NA	NA	0.20 (0.37)

* Serum androstenedione, testosterone and DHEAS were measured at Study Survey 1, 1990 – 1993; † Screening for depressive syndrome was introduced during Study Survey 2, 1993 – 1995; # Salivary cortisol levels were determined at Study Survey 4, 2002 – 2004. Values represent unadjusted means with standard deviations or unadjusted proportions. ^ Body Mass Index. ‡ Salivary cortisol level at awakening. § Salivary cortisol level 30 minutes after awakening. ‖Salivary cortisol level at 5pm. φSalivary cortisol level at bedtime. NA = Not Applicable.

$Cort_{bed}$ as well as higher androstenedione levels, was selected. Carriers of rs11119328 had a significantly higher chance of having an incident depressive syndrome: hazard ratio 1.28, 95% CI 1.03-1.59. Follow-up time consisted of 35413 person-years, with a mean follow-up time of 8.4 years per subject. 351 subjects were diagnosed with an incident depressive syndrome.

DISCUSSION

The present study shows that a common genetic variant in *HSD11B1*, rs11119328, is associated with higher late-night salivary cortisol levels and in postmenopausal women with higher androstenedione levels. This polymorphism was also related to an increased susceptibility to depression. Two other *HSD11B1* SNPs, which were in high LD with rs11119328, were related to higher cortisol levels, but not with androstenedione levels.

Several observations support the concept that 11β-HSD1 plays an important role in HPA axis regulation. In rodents, 11β-HSD1 is highly expressed at the central feedback sites of the HPA axis[5]. Harris *et al*. showed that 129/MF1 mice lacking 11β-HSD1 activity showed elevated basal corticosterone and ACTH levels and exaggerated ACTH and corticosterone responses to restraint stress, suggesting diminished glucocorticoid feedback[5]. In our study we found an association between rs11119328 and increased measures of HPA axis activity at two different point in time: higher cortisol levels at nadir (measured 1990-1993) as well as higher androstenedione levels

Table 4. Associations of *HSD11B1* SNPs and adrenal hormone levels.

	rs11119328			rs11487867			rs45487298		
	β (95% CI) *	P *	$P_{corrected}$†	β (95% CI) *	P *	$P_{corrected}$†	β (95% CI) *	P *	$P_{corrected}$†
Cortisol Measures, n = 2190#									
$Cort_{aw}$‡, $Log_{nmol/L}$	0.004 (-0.021 – 0.028)	0.78	1	0.006 (-0.018 – 0.030)	0.63	0.99	0.010 (-0.012 – 0.033)	0.38	0.98
$Cort_{aw+30}$§, $Log_{nmol/L}$	0.028 (0.005 – 0.051)	0.02	0.16	0.017 (-0.006 – 0.039)	0.15	0.73	0.020 (-0.001 – 0.042)	0.07	0.44
$Cort_{1700h}$‖, $Log_{nmol/L}$	0.027 (0.002 – 0.053)	0.03	0.27	0.029 (0.005 – 0.054)	0.02	0.18	0.030 (0.007 – 0.054)	0.01	0.10
$Cort_{bed}$ φ, $Log_{nmol/L}$	0.049 (0.019 – 0.080)	0.001	0.01	0.044 (0.014 – 0.073)	0.004	0.04	0.044 (0.016 – 0.072)	0.002	0.02
Adrenal Androgen, n = 844#									
Androstenedione, $Log_{nmol/L}$	0.039 (0.012 – 0.067)	0.005	0.04	0.016 (-0.010 – 0.042)	0.23	0.87	0.024 (-0.001 – 0.050)	0.06	0.41

* Values represent Betas, 95% Confidence Intervals and p-values adjusted for age and sex (cortisol measures) or age (androstenedione). † P-value adjusted for age and additionally corrected for multiple testing by permutation analyses. # Numbers differ slightly between analyses due to differences in genotype success rates between SNPs and differences in number of available saliva samples at different time points. ‡ Salivary cortisol level at awakening. §Salivary cortisol level 30 minutes after awakening. ‖Salivary cortisol level at 5pm. φSalivary cortisol level at bedtime.

(measured 2002-2004), indicating a higher set point of the HPA axis. One possible explanation is that this SNP is associated with lower expression of 11β-HSD1 at the central feedback sites of the HPA axis or peripherally resulting in diminished negative feedback.

Previously, only few studies evaluated the effect of *HSD11B1* SNPs on HPA axis functioning. Most of these studies were not designed to study effects on hormonal levels and were clearly underpowered to detect any significant effects[13-15]. Gambineri *et al.* found that rs12086634, which is in full LD with rs45487298 (one of the SNPs we evaluated in our study), was associated with higher dehydroepiandrostenedione sulfate (DHEAS) and a larger cortisol response to adrenocorticotropic hormone (ACTH) in women with polycystic ovary syndrome. However, they found lower morning cortisol levels, whereas we detected no effects of rs45487298 on morning cortisol levels.So far, no study evaluated the effect of *HSD11B1* SNPs on depression.

Diminished HPA axis negative feedback is thought to be a key element in depressive disorder neurobiology. So far, it is not yet clear whether these changes in HPA axis function are consequences of central neurotransmitter changes occurring during depression. Or, alternatively, that increased HPA axis activity plays a causal role in the aetiology of depression. Our study was not designed to infer causality between HPA axis activity and depression. Therefore, the observed association between rs11119328 and depression might be result of increased HPA axis activity or, alternatively, the observed increased hormonal levels might the result of a previous depression. Post-hoc analyses showed that rs11119328 was also associated with higher $Cort_{bed}$ levels in persons without a history of depression, suggesting that this association is not the result of a previous depressive episode. However, a similar trend was not observed for androstenedione.

Some other methodological issues of our study need to be discussed. First, studies using saliva sampling to determine the diurnal cortisol pattern rely heavily upon participant compliance. Samples taken in the early morning are especially sensitive to deviations from the study protocol, as cortisol levels change rapidly after awakening[17-18]. The influence of variation in sampling time is much smaller for evening cortisol levels, because these levels are much lower and more stable. Noncompliance to the study protocol has probably also influenced the salivary cortisol concentrations in our study. This might explain the negative findings for the relationship between *HSD11B1* SNPs and morning cortisol levels.

Secondly, genetic association studies are prone to both type 1 and type 2 errors. Large numbers of subjects are needed, because effect sizes for common variants are typically modest[19-20]. On the other hand, multiple testing is an issue. In our analyses we adjusted for multiple testing by using permutation analyses. However, replication studies are the golden standard and future studies are needed to confirm our results.

In summary, we showed that one tagging SNP in *HSD11B1*, rs11119328, is associated with increased late-night cortisol levels and in postmenopausal women with higher androstenedione levels. Carriers of this polymorphism are also at increased risk of developing a depressive episode. These findings suggest that 11β-HSD1 is implicated in human HPA axis regulation and depressive disorder neurobiology.

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Chapter 5

The effect of common genetic variation in 11β-hydroxysteroid dehydrogenase type 1 gene on the metabolic syndrome

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ABSTRACT

Background: The metabolic syndrome (MetS) consists of a cluster of cardiovascular risk factors, including central obesity, unfavourable lipid profile, type 2 diabetes, and hypertension. Studies suggest that 11β-Hydroxysteroid Dehydrogenase Type 1 (11β-HSD1) is involved in MetS by increasing locally available glucocorticoids in omental adipose tissue. In the present study, we wanted to evaluate the effects of common genetic variation in the 11β-HSD1 gene (*HSD11B1*) on (traits of) MetS.

Methods: Twelve SNPs including 11 tagging single nucleotide polymorphisms (SNPs) in *HSD11B1* were selected using the HapMap database. Over 6000 participants of the Rotterdam Study, a population based cohort of elderly persons, were genotyped for the selected tagging SNPs. Main outcome measures were MetS and traits of MetS, including: waist circumference, fasting glucose and triglyceride (TG) levels, high-density lipoprotein cholesterol (HDL), and diastolic and systolic blood pressure. Significant associations were replicated in the 1966 Northern Finland Birth Cohort (1966 NFBC), a population-based prospective birth cohort.

Results: After correction for multiple testing the only significant association was between rs17317033 and increased waist circumference in women (Beta 1.51 cm, [95% CI 0.61 – 2.42 cm], $P_{corrected}$ = 0.01, n = 3401). A similar trend was observed in the 1966 NFBC, however this replication study was underpowered to detect any significant effects (Beta 1.55 cm, [95% CI -0.74 – 3.84 cm], p = 0.18, n = 1136). None of the tagging SNPs was associated with an increased risk of MetS.

Conclusions: Evidence suggests that rs17317033 might be associated with increased waist circumference in women. This SNP was not related to any of the other MetS traits. None of the selected *HSD11B1* tagging SNPs was associated with an increased susceptibility to MetS.

INTRODUCTION

The metabolic syndrome (MetS) consists of a cluster of cardiovascular risk factors including, type 2 diabetes, abdominal obesity, unfavourable lipid profile, and hypertension[1]. Similarities between this syndrome and hypercortisolemic states like Cushing's syndrome suggest that glucocorticoid excess is involved in the pathogenesis of MetS[2-5]. However, although serum cortisol levels might be slightly increased in MetS, they are in the normal range and the clinical features of MetS cannot be explained by hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis alone[3, 6-9].

Glucocorticoids are centrally regulated by the negative feedback action of the HPA axis[10-11]. Next, local tissue specific cortisol levels are fine-tuned by the activity and expression of two enzymes: 11β-Hydroxysteroid Dehydrogenase Type 1 (11β-HSD1) and Type 2 (11β-HSD2). In vivo, 11β-HSD1 converts cortisone into cortisol, whereas 11β-HSD2 converts cortisol into cortisone.

In the late nineties, the possibility was raised that increased generation of cortisol in omental fat by 11β-HSD1 causes MetS. Bujalska *et al.* found that human omental fat tissue had higher 11β-HSD1 activity than subcutaneous fat[12]. Moreover, the expression of this enzyme, and the differentiation of preadipocytes to adipocytes, was increased further after exposure to cortisol. This suggested that visceral adipose 11β-HSD1 expression could drive central obesity, one of the key features of MetS. This hypothesis gained further support after the observation that transgenic mice overexpressing 11β-HSD1 in adipose tissue had increased levels of corticosterone (the equivalent of cortisol in rodents) and developed visceral obesity, as well as other components of MetS, such as insulin resistant diabetes and hyperlipidemia[13]. Since then 11β-HSD1 has been a target for drug research, which has led to the recent discovery of Rosenstock *et al*. who showed that the 11β-HSD1 inhibitor INCB13739 is successful in reducing fasting glucose and HbA1C levels when added to metformin monotherapy[14].

Previously, we explored the effects of candidate single nucleotide polymorphisms (SNPs) in the 11β-HSD1 gene (*HSD11B1*), 83,557insA (rs45487298), on body composition, lipid profile, glucose metabolism, and blood pressure[15]. This SNP was an interesting candidate SNP because it showed lower 11β-HSD1 expression in vitro and initial studies reported associations with cortisone reductase deficiency, polycystic ovary syndrome, type 2 diabetes and hypertension[16-19]. However, those findings could not be replicated in larger population studies including our own[20-22]. Although these results might discourage further *HSD11B1* candidate gene studies, large regions of this gene remained unexplored. Genetic variation is common in this region, and up to now over 500 genetic variants have been detected in the *HSD11B1* gene region (db SNP database, build 132).

In the present study we wanted to explore the effects of common genetic variation in *HSD11B1* on features of the MetS using a tagging SNP approach. For this study we used data from the Rotterdam Study, a population-based cohort in the elderly[23]. Significant associations were replicated in the Northern Finland Birth Cohort 1966 (NFBC 1966), a population-based birth cohort from the 16th gestational week into adulthood[24].

METHODS

Study populations

The Rotterdam Study is a population-based, prospective cohort study of men and women aged 55 years and over[25]. All residents aged 55 or older of Ommoord, a district of Rotterdam, The Netherlands, were invited to participate. A total of 7983 men and women (78% of those eligible) entered the study. The Rotterdam Study was approved by the medical ethics committee of the Erasmus MC and written informed consent was obtained from all participants. Baseline examinations, including a home interview and an extensive physical examination at the research centre, took place between 1990 and 1993. For the current study, we used baseline examinations and measurements of the third study survey, which took place between 1997 and 1999. *HSD11B1* genotype data were available of 6508 participants.

Significant associations were replicated in the NFBC 1966. This is a population-based prospective birth cohort, which recruited mothers with expected dates of delivery in 1966 in the two northernmost provinces of Finland through maternity health centres, 12,058 live births (96.3% of those eligible) were included in the study[24]. Follow-up in 1997-1998 consisted of questionnaires sent to all participants at age 31 years, and clinical examinations were conducted for those living in the original target region or in the Helsinki area (n = 8463 eligible). 71% of those eligible (n = 6007) had a clinical examination, all of whom provided written informed consent. The study was approved by the University of Oulu ethics committee.

Assessment of metabolic parameters and waist circumference

Outcome measures were selected on the basis of criteria of MetS of the National Cholesterol Education Program / Adult Treatment Panel III (NCEP/ATP III), and included waist circumference, fasting glucose levels, serum triglycerides (TG), serum HDL and systolic and diastolic blood pressure[1].

In the Rotterdam Study, the waist circumference was measured at the level of the umbilicus (n = 5942). Blood pressure was measured in a sitting position at the right upper arm with a random-zero sphygmomanometer (n = 6304). At baseline, non-fasting blood samples were drawn, HDL levels (n = 6361) were determined and DNA was extracted. During the third study survey, fasting glucose (n = 3549) and TG (n = 3445) were measured additionally. Information on current medication use was assessed at baseline and at all study waves. Subjects using glucose lowering medication (n = 166), blood pressure lowering drugs (n = 2017) and serum lipid reducing agents (n = 157) and with missing data on these drugs (n = 878, n = 3 and n = 1792, respectively) were excluded from the analysis with regard to fasting glucose levels, blood pressure and lipid levels respectively.

In the NFBC 1966, all traits were measured at the 31-years clinical examination. Waist circumference was measured midway between the lowest rib margin the iliac crest. Blood pressure was measured twice in a sitting position from the right arm after 15 minutes rest. Fasting blood samples were drawn for DNA extraction, and for analyses of fasting glucose, HDL, and TG levels[26].

Metabolic Syndrome

MetS was diagnosed if participants met the current NCEP/ATP ШI criteria[1]. These include presence of any three of the following five traits: abdominal obesity, defined as a waist circumference in men > 102 cm and in women > 88 cm; serum TG ≥ 1.7 mmol/L or lipid lowering medication; serum HDL < 1 mmol/L in men and < 1.3 mmol/L in women or drug treatment for low HDL; a systolic blood pressure ≥ 130 mmHg, a diastolic blood pressure ≥ 85 or drug treatment for elevated blood pressure and fasting glucose ≥ 5.6 mmol/L or drug treatment for elevated blood glucose. In the Rotterdam Study, presence or absence of the MetS was determined at study survey three. 881 persons were excluded because data on MetS traits or medication use were missing.

DNA analysis

Tagging SNPs in *HSD11B1* were selected using the HapMap database (Public Release #20). The following selection criteria were used: CEU samples, unrelated only, allele frequency cut-off of 5%, R^2 threshold for clusters of 0.8, minimal genotype coverage of SNPs to be tag SNPs of 85% and minimal genotype coverage of SNPs to be clustered of 70%. SNPs were selected to tag variation 5kb upstream and 1kb downstream *HSD11B1*. This resulted in the selection of 11 tagging SNPs. Since *HSD11B1* 83,557insA (rs45487298), which is in full LD with a T > G substitution 40 bp downstream at position 83,597 (rs12086634), has been extensively studied previously, we also included this polymorphism in our analyses.

In the Rotterdam Study, the appropriate Assay-by-Design mixes were designed, synthesized and supplied by Applied Biosystems (Foster City, CA, USA). See Table 1 of Chapter 4 for primer sequences. Five-µl PCR reactions containing ~10 ng of DNA, 0.0625 µl of 80x Assay-by-Design mix, 2.4375 µl water, and 2.5 µl Universal Master Mix (Applied Biosystems) were performed in 384-well plates. The reaction conditions were: 2 min 50° C, 10 min 95 ^{0}C, followed by 40 cycles of 15 sec 92 ^{0}C, and 60 sec 60 ^{0}C. Plates were analyzed using the Applied Biosystems 7900HT Sequence Detection System and SDS version 2.0 software (Applied Biosystems). To confirm the accuracy of genotyping results, 5% of the samples were re-genotyped with the same method. Percentage of concordance was higher than 99% for all genotyped variants.

NFBC 1966 was genotyped using the Illumina Infinium Human CNV – 370DUO array. After applying exclusion criteria and quality control procedures, information on 329,091 SNPs was available in 4,763 individuals. Details on these procedures and genotyping methods have been described previously[27]. Non-genotyped SNPs (rs17317033) were imputed using IMPUTE version 1.0 using the CEU HapMap sample[28-29]. Genotypes were only called if the probability of the genotype was 90% or greater.

Statistical Analysis

Hardy-Weinberg Equilibrium (HWE) was calculated according to standard procedures using Chi-square analyses. Haploview (release 4.1) was used to determine the Linkage Disequilibrium (LD) blocks and the r-squared values between the polymorphisms.

Linear regression analyses models were used to study the associations between genetic markers and MetS traits. Outcome measures were normalised when necessary to optimise normal distribution. Analyses were adjusted for age and sex, and stratified for gender only for those features of the metabolic syndrome for which the NCEP/ATP III makes a distinction between men and women: waist circumference and HDL.

The effects of *HSD11B1* SNPs on the prevalence of MetS were evaluated using logistic regression models, adjusted for age and sex and additionally stratified for gender. These analyses were performed using SPSS for Windows, release 15.0. P-values were corrected for number of tested SNPs using a permutation procedure (10,000 permutations) available in the R package GenABEL version 1.6-4[30]. Associations that remained significant after correction for multiple testing were replicated in 1966 NFBC. Post-hoc, a meta-analysis was performed to combine the results of the Rotterdam Study and 1966 NFBC using the package 'meta'[31] running for 'R'[32]. Given that no heterogeneity was observed based on the Q-test, a fixed-effects model was used to weight the betas.

RESULTS

Characteristics of the participants of the Rotterdam Study are given in Table 1. 12 SNPs including 11 tagging SNPs were selected. All SNPs were in HWE. Fig. 1 shows a schematic overview of *HSD11B1*, the rs numbers of the selected SNPs and the observed minor allele frequencies. The linkage disequilibrium plot, showing the LD blocks and the r-squared values between all polymorphisms are displayed in Fig. 3 of Chapter 4. Raw genotype frequencies, call rates, HWE and minor allele frequencies (MAF) of the genotype results in the Rotterdam Study are given in Table 2 of Chapter 4.

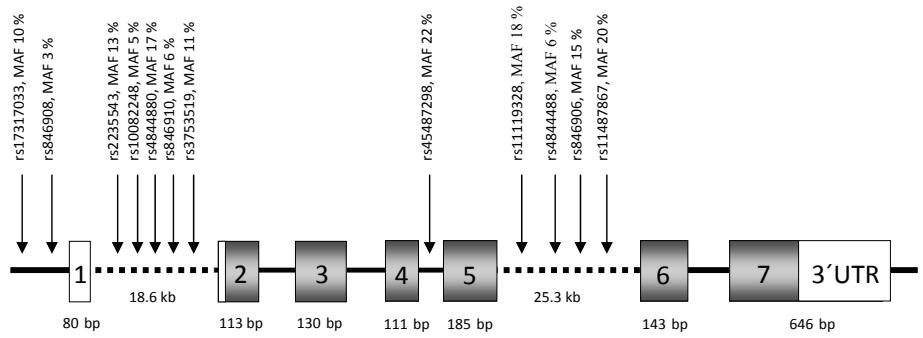
Table 2 shows the results of linear regression analyses with *HSD11B1* tagging SNPs and MetS traits in the Rotterdam Study. Only those associations with p-values (not corrected for multiple testing) below 0.05 are presented. Taking the number of analysed SNPs into account, the only significant relation was between rs17317033 and increased waist circumference in women (Beta 1.51, [95% CI 0.61 – 2.42], n = 3401, see also Table 2). A similar trend was observed in the 1966 NFBC (Beta 1.55, [95% CI –0.74 – 3.84], p = 0.18). However, due to weak LD with the adjacent SNPs on the Illumina array, this genotype could only be predicted with a $P > 0.9$ in 1136 women of the 1966 NFBC, and therefore this replication cohort was probably underpowered to detect any significant effects. A post-hoc meta-analysis showed that the weighed Beta was 1.52 cm (95% CI 0.68 – 2.36 cm, p = 0.0004).

Table 1. Characteristics of participants from Rotterdam Study.

Variable	Rotterdam Study	
	n	percentage or mean (SD)†
Sex, %		
Male	2640	40.6
Female	3868	59.4
Age, years		
Study wave 1	6508	69.5 (9.1)
Study wave 3	3917	72.8 (7.4)
Waist, cm[1]		
Male	2457	94.3 (9.5)
Female	3485	87.8 (11.5)
Triglycerides, mmol/L[3]	3445	1.5 (0.73)
HDL‡, mmol/L[1]		
Male	2599	1.2 (0.33)
Female	3762	1.4 (0.37)
Lipid lowering Drugs, %[1]		
No	4412	69.4
Yes	157	2.5
Missing	1792	28.1
Blood Pressure, mmHg[1]		
Systolic	6304	139.3 (22.3)
Diastolic	6303	73.7 (11.5)
Antihypertensives, %[1]		
No	4284	68.0
Yes	2017	32.0
Missing	3	0.0
Fasting glucose, mmol/L[3]	3549	5.9 (1.4)
Antidiabetics, %[3]		
No	2505	70.6
Yes	166	4.7
Missing	878	24.8
Metabolic syndrome, %[3]		
No	1442	36.8
Yes	1594	40.7
Missing	881	22.5

†Values represent unadjusted means with standard deviations or unadjusted proportions. ‡High-density lipoprotein cholesterol. [1]Data presented are measured at study survey 1. [3]Data presented are measured at study survey three.

None of the *HSD11B1* SNPs was associated with MetS, diagnosed using the NCEP/ATP Ш criteria. Stratification for gender did not reveal any significant associations either (data not shown, p-values not corrected for multiple testing > 0.05 for all tests).



MAF = minor allele frequency, bp = base pairs, kb = kilobase.

Figure 1. Schematic overview of *HSD11B1*, selected tagging SNPs and minor allele frequencies.

DISCUSSION

The present study shows that common genetic variation in *HSD11B1* is not associated with susceptibility to MetS. In the Rotterdam Study, rs17317033 was related to a larger waist circumference in women. A similar trend was observed in the 1966 NFBC, however this study was underpowered to detect any significant effects. None of the of *HSD11B1* SNPs was associated with other features of the MetS after correction for multiple testing.

Recently, Miyamoto *et al*. screened 48 Japanese persons for *HSD11B1* SNPs and related those SNPs to MetS in an urban Japanese Study population[33]. None of the selected SNPs were associated with susceptibility to MetS after taking multiple testing into account. One SNP showed a trend towards a higher risk of MetS, however this polymorphism was not associated with any of the independent MetS traits. We cannot compare the results this study with our observation that rs17317033 was associated with increased waist circumference, because this polymorphism is monomorphic in Asian populations. Moreover, this study did not evaluate genetic variation upstream exon 1.

Previously, we reported no effect of *HSD11B1* 83,557insA (rs45487298) and R453Q in the hexose-6-phosphate dehydrogenase gene on blood pressure, glucose metabolism, and body composition[15]. Recently, we evaluated the effects of *HSD11B1* tagging SNPs and measures of HPA axis activation and depression (Chapter 4). We showed that rs11119328 was associated with higher salivary cortisol levels, increased serum levels of androstenedione in women and higher risk of an incident depressive disorder. We hypothesized that rs11119328 is (or tags) an inactivating variant, which leads to lower expression of 11β-HSD1 at the central feedback sites of the HPA axis, resulting in diminished negative feedback. In the present study, we did not

Table 2. Associations of *HSD11B1* SNPs with components of the Metabolic Syndrome.

Outcome Measures *NCEP/ATP III* Criteria*	rs number	n	Mean (95% CI†)	P°	$P_{corrected}$¶
Waist circumference, cm *Male*	*No significant associations*				
Waist circumference, cm *Women*	rs17317033				
	WT‡	2776	87.6 (87.2 – 88.0)	0.001	0.01
	HZ§	594	88.9 (88.0 – 89.8)		
	HO‖	31	92.3 (88.4 – 96.3)		
	rs2235543				
	WT	2555	87.6 (87.2 – 88.0)	0.03	0.25
	HZ	748	87.7 (88.0 – 89.3)		
	HO	61	86.3 (88.4 – 91.9)		
	rs4844488				
	WT	2555	88.0 (87.6 – 88.4)	0.02	0.20
	HZ	748	87.0 (85.8 – 88.1)		
	HO	61	80.7 (74.1 – 87.3)		
Fasting glucose, mmol/L	*No significant associations*				
Triglycerides, mmol/L	rs2235543				
	WT	1565	1.49 (1.46 – 1.53)	0.03	0.24
	HZ	508	1.55 (1.48 – 1.61)		
	HO	36	1.65 (1.42 – 1.88)		
	rs4844880				
	WT	1433	1.48 (1.45 – 1.52)	0.006	0.06
	HZ	605	1.57 (1.51 – 1.62)		
	HO	64	1.56 (1.39 – 1.74)		
	rs846910				
	WT	1831	1.50 (1.46 – 1.53)	0.02	0.16
	HZ	230	1.59 (1.50 – 1.68)		
	HO	1	1.60 (NA»)		
	rs3753519				
	WT	1665	1.49 (1.46 – 1.52)	0.03	0.26
	HZ	401	1.59 (1.52 – 1.66)		
	HO	25	1.36 (1.09 – 1.64)		
HDL#, mmol/L	rs11487867				
Male	*WT*	990	1.18 (1.16 – 1.20)	0.05	0.33
	HZ	491	1.23 (1.20 – 1.26)		
	HO	59	1.21 (1.12 – 1.29)		
	rs45487298				
	WT	965	1.18 (1.16 – 1.20)	0.02	0.16
	HZ	550	1.23 (1.20 – 1.26)		
	HO	72	1.20 (1.12 – 1.28)		
HDL#, mmol/L *Women*	*No significant associations*				
Systolic blood pressure, mmHg	*No significant associations*				
Diastolic blood pressure, mmHg	rs10082248				
	WT	3705	73.3 (72.9 – 73.6)	0.03	0.21
	HZ	385	74.4 (73.3 –75.6)		
	HO	12	76.6 (70.3 – 82.8)		

*NCEP/ATP III = National Cholesterol Education Program / Adult Treatment Panel III. ‡WT = Wildtypes. §HZ = Heterozygotes. ‖HO = Homozygotes. # HDL = high-density lipoprotein cholesterol. †Values represent adjusted means and adjusted 95% Confidence Intervals. °P = p-value, adjusted for age (analyses stratified for sex) or age and sex (unstratified analyses). ¶$P_{corrected}$ = p-value, additionally corrected for multiple testing. »NA = not applicable.

detect any significant effects of rs11119328 on any of the MetS traits. Possibly, increased HPA axis activity compensates for an attenuated generation of cortisol at the tissue level. Alternatively, the detected associations with higher HPA axis parameters are false-positive associations.

Previously, several studies suggested that 11β-HSD1 might be implicated in the aetiology of MetS and proposed 11β-HSD1 as in interesting drug target for treatment of MetS traits[2, 4, 34]. Recently, Rosenstock *et al*. confirmed these hypotheses and showed that when the 11β-HSD1 inhibitor INCB13739 was added to metformin monotherapy in type 2 diabetes patients exhibiting inadequate glycemic control, significant reductions of fasting glucose and HbA1C levels were achieved compared to a placebo group. Moreover, this drug was well tolerated, body weight was decreased, and in hyperlipidemic patients total cholesterol (TC), low-density lipoprotein cholesterol (LDL) and TG were all significantly reduced[14]. Our results confirm that 11β-HSD1 might influence body composition. However, no significant associations were observed between the other components of the MetS, such as lipid profile and glucose metabolism.

How should we relate the results of the INCB13739 trial to the observed negative results of our genetic association study? Negative genetic association studies do not prove that a protein is not involved in the pathogenesis of a disease. Possibly, common genetic variation in *HSD11B1* does not result in functional alterations of the gene. Up to now 503 genetic variants have been identified in the *HSD11B1* gene region, but only 7 polymorphisms are located in the translated regions of *HSD11B1*[35]. Five of those variants are synonymous SNPs, which do not cause amino acid changes. The two other mutations include a missense mutation in exon 2 (rs111629669) resulting in a threonine to isoleucine change and a frame shift mutation in exon 7 (rs34727634). The minor allele frequencies of both variants are unknown, which suggests that they are probably rare variants. In our study we used an allele frequency cut-off of 5%, which means that with the selection of our SNPs we are probably unable to detect the effects of rare functional genetic variants.

Some other methodological issues of our study need to be discussed. Multiple testing is an issue in genetic association studies because they typically involve multiple genetic markers and outcomes. In our study we evaluated the effect of 12 SNPs on 9 phenotypes (MetS, waist circumference and HDL levels in men and women separately, fasting glucose and TG levels, and diastolic and systolic blood pressure) resulting in 108 tests. To correct for the number of tested SNPs we used permutation analyses, and only associations that remained significant after this correction were replicated in 1966 NFBC. However, by increasing the significance threshold, false-negative findings might be introduced. Possibly, one or more associations with uncorrected p-values <0.05 may reflect true positive relationships.

Another issue of our study is that in our elderly study population a large proportion of participants used antihypertensives, antidiabetics or lipid lowering drugs. These persons were excluded in the separate blood pressure, fasting glucose and serum lipid analyses respectively. This is problematic, because by doing so we preferentially exclude persons with an unfavourable metabolic profile and undermine the external validity of our study results. However, in the

analyses of waist circumference, the key feature of the MetS, those persons were not excluded. Moreover, medication use is part of the definition of MetS, and therefore contributed to the analyses of *HSD11B1* SNPs and susceptibility to MetS.

In conclusion, evidence suggests that rs17317033 is associated with a larger waist circumference in women. However, none of the *HSD11B1* tagging SNPs was consistently related to any of the other MetS traits or with presence of MetS. This indicates that common genetic variation in *HSD11B1* does not contribute to susceptibility to MetS in an elderly Caucasian population.

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Chapter 6

Salivary cortisol is related to atherosclerosis of carotid arteries: the Rotterdam Study

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ABSTRACT

Background: Dysregulation of the hypothalamic-pituitary-adrenal (HPA-axis) has been suggested as an independent risk factor for ischemic heart disease. The aim of our study was to evaluate whether two markers of the HPA-axis activity, the level of salivary cortisol and the diurnal salivary cortisol pattern, are associated with atherosclerosis of the carotid arteries in an elderly population.

Methods: 1866 participants of the Rotterdam Study, a population-based cohort study in the elderly, provided four salivary cortisol samples throughout one day and underwent ultrasonography to examine the presence of plaques in the common, internal and bifurcation sites of both carotid arteries. Two summary measures of the separate cortisol values were computed: area under the curve (AUC), which is a measure of total cortisol exposure while awake, and the slope, which is a measure of diurnal cortisol decline.

Results: Total cortisol exposure while awake (AUC) was associated with higher plaque scores ($\beta = 0.08$ per SD of AUC, 95% CI; 0.00 – 0.17, p-value = 0.04) in a fully adjusted linear regression model. Persons with an AUC in the highest tertile had a higher number of plaques of carotid arteries compared to those in the lowest tertile (3.08 versus 2.80, 95% CI of difference; 0.09 – 0.48, p-value = 0.005). There was no relation between diurnal cortisol decline and plaque score.

Conclusion: Our results support the hypothesis that increased total cortisol exposure is independently associated with atherosclerosis of the carotid arteries.

INTRODUCTION

For several decades, chronic exposure to psychosocial stressors has been implicated as a risk factor for cardiovascular disease (CVD). For instance, lack of social support, low socio-economic status, unhappy marriages and work stress are consistently associated with an increased incidence of CVD in several epidemiological studies[1-5].

One of the possible biological mechanisms through which chronic stress may influence the risk of CVD is dysregulation of the hypothalamic-pituitary-adrenal axis (HPA-axis). In healthy persons, the HPA-axis shows a diurnal rhythm with peak cortisol levels approximately 30 minutes after awakening, declining cortisol levels throughout the day and a nadir around midnight[6]. In response to physical or psychosocial stress (e.g. physical exercise, having a lunch meal or emotional arousal) the HPA-axis gets activated and as a result short-term cortisol increases are observed[7-8].

Exposure to chronic stressors might dysregulate two different aspects of the HPA-axis activity. First, overall levels of cortisol can be increased. This is observed during periods of perceived work stress[9], in men with lower social economic status[10] and in men with overall negative affect[11]. Secondly, a smaller decline of cortisol levels throughout the day, a flatter slope, is seen in persons with marital problems[12], trait negative affect[11], low social economic status[13], unemployed individuals with high financial strain[14] and women undergoing a divorce or separation[15].

Elevated cortisol levels have numerous negative health consequences throughout the human body. Cortisol is implicated in the regulation of the immune system, glucose and lipid metabolism and maintenance of cardiac output by increasing vascular tone and decreasing vascular permeability. Dysregulation of the HPA-axis is associated with hypertension[16-17], increased heart rate and increased levels of total and LDL cholesterol and fasting insulin and glucose[18].

Despite the strong hypothetical basis for a causal relationship between cortisol and CVD and more specifically the development of atherosclerosis, only one large epidemiological study has tested this hypothesis. Matthews *et al.*[19] showed that flatter cortisol slopes, but not average cortisol levels, correlate with the presence of any coronary calcification in young and middle-aged adults. Although this is an important finding, the prevalence of atherosclerosis was very low in this relatively young age group. The aim of the present study was to evaluate whether HPA-axis regulation is associated with atherosclerosis in the elderly. We tested the hypothesis that total cortisol secretion throughout the day and diurnal cortisol decline, correlate with atherosclerosis of the carotid arteries using data from the Rotterdam Study, a population-based study in an elderly population.

METHODS

Study Population

This investigation is embedded in the Rotterdam Study, an ongoing population-based cohort study aimed at assessing risk factors for chronic diseases in the elderly[20]. In 1990, all inhabitants of a suburb of Rotterdam aged 55 years and over were invited and 7983 agreed to participate (response 78%). The Rotterdam Study was approved by the Medical Ethics Committee of the Erasmus MC and written informed consent was obtained from all participants. Assessment of the salivary diurnal cortisol rhythm was added to the fourth survey study protocol, which took place between January 2002 and December 2004.

Of the 3550 persons who participated in the fourth survey, 2569 (74 %) returned one or more saliva samples. We excluded 434 persons because they had missing data on salivary cortisol levels or collection times or salivary cortisol levels above the 98th percentile, 4 persons because they did not collect saliva correctly, 29 persons because they did not undergo ultrasonography of the carotid arteries and 236 persons because they were using glucocorticoid applications. The final population for analysis consisted of 1866 persons with complete data on ultrasonography and at least one summary cortisol measure: the slope.

Measures of Atherosclerosis

Ultrasonography of both carotid arteries was performed with a 7.5-MHz linear-array transducer and a duplex scanner (Acuson 128, Siemens and Esaote, Pie Medical). Both left and right internal carotid artery, carotid bifurcation, and common carotid artery were examined for the presence of plaques. Plaques were defined as a focal widening relative to adjacent segments with the protrusion into the lumen. The total plaque score reflected the number of sites with plaques ranging from 0-6[21].

Assessment of Covariates

During the home interview of the fourth study visit, information on current smoking status, medication and educational attainment was collected. We defined 8 categories of educational attainment: 1 = primary education, 2 = primary education plus a higher not completed education, 3 = lower vocational education, 4 = lower secondary education, 5 = intermediate vocational education, 6 = general secondary education, 7 = higher vocational education and 8 = university. In the Netherlands, educational attainment is the best measure for socio-economic status (SES). In the Dutch elderly population, income is not the best proxy for differences in SES, because in this country retirement insurances level out differences in income. The same holds for 'last occupation' or 'highest occupation', because in this generation, it was very common for women to stay at home regardless of SES. Depressive symptoms were evaluated using the validated Dutch version of the Center for Epidemiologic Studies Depression Scale (CES-D)[22]. Additionally, clinical measures were obtained at the research centre. Detailed information on all glucocorticoid

applications (including systemic glucocorticoids, intra-articular steroid injections, steroid creams and inhalants), aspirin and statin use and blood pressure lowering medication was obtained from one or more of the seven pharmacies serving the research area. Nearly all participants (95%) were registered at one of these pharmacies, which are fully automated, and linked to the database of the Rotterdam Study. Fasting blood samples were drawn and total cholesterol, high-density lipid-cholesterol (HDL cholesterol) and glucose were determined. We defined diabetes mellitus as a fasting glucose of 7.0 mmol/L or greater, a non-fasting glucose of 11.1 mmol/L or greater or the use of blood glucose lowering medication. Blood pressure was measured at the right brachial artery using a random-zero sphygmomanometer with the participant in sitting position. The body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

Salivary Cortisol Protocol

All participants who came to the research centre during the fourth study survey were asked to collect saliva samples at home using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). Participants received detailed oral and written instructions concerning the saliva sampling. They were told to collect four saliva samples during one single weekday at home: directly after awakening ($Cort_{aw}$), 30 minutes later ($Cort_{aw+30}$), at 5 pm ($Cort_{5pm}$) and at bedtime ($Cort_{bed}$) and to write down the exact times and date of saliva collections. Furthermore, they were asked not to brush their teeth and not to eat 15 minutes before saliva sampling to avoid contamination of saliva with blood caused by micro-injuries to the oral cavity. Besides these restrictions, subjects were otherwise free to follow their normal daily routines on the sampling day. One day after the saliva collections, the Salivettes were returned at the research centre. Samples were stored in the freezer at minus 80° C until completing the fourth study survey and sent to the laboratory of Biopsychology, TU Dresden, Germany. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and inter-assay coefficients of variation were below 6% and 9%, respectively. The lower limit of detection was 0.4 nmol/L. All, but two of the saliva samples had detectable levels of cortisol.

Salivary Cortisol Analysis

Data were screened for quality of cortisol measurements. Due to sampling errors 26 samples were excluded from the analyses: 1 because it was clearly contaminated with blood, two because the label on the Salivette was unreadable, 23 because those Salivettes were most likely swapped by the participants and 2 because the Salivettes did not contain saliva.

For each time point, cortisol values that were above the 98^{th} percentile in the original cortisol dataset, were excluded from the final dataset, in order to normalise the distribution of cortisol measurements and to exclude misclassification due to possible measurement errors ($Cort_{aw}$ 46 were excluded; $Cort_{aw + 30}$ 46 were excluded; $Cort_{5pm}$ 45 were excluded; $Cort_{bed}$ 47 were excluded). After this exclusion, cortisol levels followed a normal distribution. This conservative

cut-off was chosen due to the high number of outliers. However, using the 99th percentile as a cut-off for outliers did not essentially change the results presented in this paper.

All analyses reported were performed on each of the four separate cortisol measurements. However, the main focus of our study was to evaluate the effects of two markers of the HPA-axis activity: the area under the curve (AUC), which is a measure of total cortisol exposure while awake, and the slope, which is a measure of diurnal cortisol decline. The AUC was determined by the total area under the curve given by the cortisol measurements in nmol/L on the y-axis and the time between the cortisol measurements on the x-axis. To correct for differences in hours being awake, the AUC was divided by number of hours between the first cortisol measurement (at awakening) and the last cortisol measurement (before going to bed). The AUC was computed only for those who collected four saliva samples and had complete data on collection times at all time points. In this way, we obtained data on the AUC of 1963 participants.

The slope was calculated by fitting a linear regression line for each participant that predicted the cortisol values from time since awakening. The second sample was excluded to minimize the impact of the morning rise on the estimation of slopes. The final dataset consisted of 2016 participants with data on the slope.

Statistical Analysis

We compared persons with and without plaques for known risk indicators for cardiovascular disease: sex, age, smoking status, systolic and diastolic blood pressure, BMI, total cholesterol, HDL cholesterol, diabetes mellitus, blood pressure lowering medication, aspirin and statin use and educational attainment using the Student's t-test for continuous variables and Pearson's Chi-square for the categorical variables. Next, we examined the associations of these risk factors with two summary measures of cortisol, AUC and slope using linear regression models.

Linear regression models were used to study the associations between the cortisol measures and the plaque score. First, we adjusted the analyses for age, sex, time between awakening and saliva collection in the analyses of the separate cortisol measures and for age, sex, time between second and third measurement in the analyses of the AUC, to adjust for differences in timing of the sleep-wake cycle (model 1). Second, we adjusted the analyses as for model 1 and additional confounders: current smoking status, month of saliva collection, blood pressure lowering medication and aspirin and statin use (model 2). Finally, we additionally adjusted the analyses for possible mediators: educational attainment, systolic blood pressure, diabetes mellitus, BMI and total and HDL cholesterol (model 3). We did not include depressive symptoms in our models, because adjustment did not change effect estimates (changes < 0.05 %).

Next, we divided the AUC and slope distributions into tertiles, and studied the differences in mean plaque score using ANOVA, and risk of having any atherosclerotic plaques using logistic regression analysis, between the tertiles in fully adjusted models. Missing information on covariates was imputed by means. Data are presented as betas (β) and 95% CI, as means and 95% CI or as odds ratios and 95% CI. Data were analysed using SPSS for Windows, release 12.0.1.

RESULTS

The baseline characteristics for participants with and without atherosclerotic plaques are given in Table 1. Overall, a higher percentage of men had plaques and persons with plaques were more often smokers. They were older, had higher systolic blood pressure, lower HDL cholesterol and used more often statins, aspirin and antihypertensives. Next, we examined the associations of the baseline characteristics with AUC and slope (Table 2). Smoking, systolic blood pressure, diabetes mellitus, educational attainment and presence of plaques correlated positively with AUC. Higher age, current smoking and higher BMI were associated with flatter slopes. There were no associations with the summary measures AUC and slope for the other baseline characteristics.

Table 3 shows the associations of the cortisol measures with continuous measure of plaque score. Using the age and sex adjusted model, we found that per 10 nmol/L higher cortisol 30 minutes after awakening, the plaque score increased by 0.09 ($\beta = 0.09$, 95% CI; 0.00 – 0.18, $p = 0.05$). We also observed a significant relation between $Cort_{5pm}$ and $Cort_{bed}$ with higher plaque scores ($\beta = 0.40$, 95% CI; 0.11 – 0.69, $p = 0.006$ and $\beta = 0.51$, 95% CI; 0.06 – 0.97, $p = 0.03$ respectively). The main focus of our study, however, was on the two summary cortisol measures. The AUC was significantly associated with plaque score ($\beta_{per\ SD\ of\ AUC} = 0.12$, 95% CI; 0.04 – 0.20, $p = 0.005$ respectively). We could not detect an association between slope and plaque score. Adjustment for possible confounders attenuated the observed relations moderately: the association between $Cort_{aw+30}$ and $Cort_{bed}$ and plaque score did not reach significance anymore ($\beta = 0.06$, 95% CI; -0.03 – 0.15, $p = 0.20$ and $\beta = 0.24$, 95% CI; -0.21 – 0.68, $p = 0.30$ respectively). However, the associations with $Cort_{5pm}$ and AUC remained statistically significant ($\beta = 0.35$, 95% CI; 0.07 – 0.64, $p = 0.01$ and $\beta = 0.08$, 95% CI; 0.00 – 0.17, $p = 0.04$ respectively, Table 3). Additional adjustment for possible mediators changed the results only marginally (Table 3).

To get a better understanding of what the risk of having any atherosclerotic plaques or more severe atherosclerosis of carotid arteries was for subjects with low, intermediate or high total cortisol exposure and diurnal cortisol decline, we collapsed the AUC and slope distributions into tertiles. Table 4 shows that participants with AUC values in the highest tertile had more atherosclerotic plaques compared to those in the lowest tertile in a fully adjusted model (3.08 versus 2.80, 95% CI of difference; 0.09 – 0.48, p-value = 0.005). There was no significant difference in plaque score between the middle and lowest tertile. Next, we performed logistic regression analyses to study the risk of having one or more atherosclerotic plaques for the different tertiles of AUC and slope. We could not detect a statistically significant difference between tertiles of AUC and presence of atherosclerotic plaques. There were also no significant relations between tertiles of slope and plaque score and presence of atherosclerotic plaques.

Table 1. Characteristics of Study Population according to presence or absence of atherosclerotic plaques.

Variable	Plaques yes* (n = 1617)	Plaques no* (n = 249)	P-value
Sex			
male, %	45.5	26.9	<0.001
female, %	54.5	73.1	
Age, years (SD)	75.1 (5.7)	73.0 (5.0)	<0.001
Smoking			
current, %	11.9	6.6	<0.001
previous, %	58.5	47.1	
never, %	29.6	46.3	
Systolic Blood pressure (mm Hg) (SD)	153.6 (21.7)	147.0 (19.3)	<0.001
Diastolic Blood pressure (mm Hg) (SD)	79.5 (11.2)	80.7 (10.2)	0.10
BMI ($kg*m^{-2}$) (SD)	27.4 (3.9)	27.7 (4.6)	0.28
Total Cholesterol (mmol/L)	5.59 (1.0)	5.60 (1.0)	0.86
HDL Cholesterol (mmol/L)	1.42 (0.4)	1.52 (0.4)	<0.001
Diabetes Mellitus			
Yes, %	13.7	10.1	0.15
No, %	86.3	89.9	
Plaque Score (SD)	3.4 (1.6)	0	NA
Depressive Symptoms, CES-D‡, (SD)	5.9 (7.0)	5.7 (7.7)	0.73
Educational Attainment			
Primary Education, %	13.3	13.4	0.13
Higher not Completed Education, %	12.4	16.7	
Lower Vocational Education, %	17.8	17.5	
Lower Secondary Education, %	12.6	15.4	
Intermediate Vocational Education, %	28.2	19.5	
General Secondary Education, %	3.3	4.5	
Higher Vocational Education, %	11.2	11.8	
University, %	1.3	1.2	
Use of Aspirins			
Yes, %	23.5	13.3	<0.001
No, %	76.5	86.7	
Use of Statins			
Yes, %	19.4	6.4	<0.001
No, %	80.6	93.6	
Blood pressure lowering medication			
Yes, %	46.1	29.3	<0.001
No, %	53.9	70.7	
Salivary Cortisol (nmol/L) (SD)			
At awakening	14.5 (7.6)	14.3 (7.9)	0.80
30 min after awakening	18.2 (9.2)	17.3 (8.5)	0.16
At 5 pm	4.2 (2.9)	3.6 (2.3)	0.008
Before going to bed	2.2 (1.8)	1.9 (1.6)	0.04
Area Under the Curve (AUC) †	8.2 (3.5)	7.7 (3.2)	0.02
Slope	-0.82 (0.5)	-0.83 (0.5)	0.75

*Values represent unadjusted means with standard deviations or unadjusted proportions. †Analyses with AUC are adjusted for time between second and third salivary cortisol measurement. ‡ CES-D = Center for Epidemiologic Studies Depression Scale.

Table 2. Associations of Covariates with Summary Cortisol Measures.

	Summary Cortisol Measures					
	Area Under the Curve*, nmol/L			Slope†, nmol/L/hour		
Covariates	β	95% CI	P-value	β	95% CI	P-value
Age, years	0.011	-0.017 – 0.039	0.46	0.007	0.003 – 0.011	0.002
Sex, female	-0.179	-0.501 – 0.142	0.27	0.040	-0.007 – 0.088	0.10
Current Smoking, Yes	1.159	0.647 – 1.670	<0.001	0.112	0.036 – 0.187	0.004
Systolic Blood pressure, mm Hg	0.008	0.000 – 0.015	0.04	-0.000	-0.001 – 0.001	0.54
Diastolic Blood pressure, mm Hg	-0.004	-0.018 – 0.010	0.60	-0.000	-0.002 – 0.002	0.80
BMI, $kg*m^{-2}$	-0.028	-0.068 – 0.013	0.18	0.007	0.001 – 0.013	0.03
Total Cholesterol, mmol/L	-0.158	-0.318 – 0.002	0.05	0.006	-0.018 – 0.030	0.61
HDL Cholesterol, mmol/L	0.259	-0.164 – 0.682	0.23	-0.004	-0.067 – 0.058	0.89
Diabetes Mellitus, Presence	0.865	0.366 – 1.363	0.001	-0.023	-0.096 – 0.050	0.54
Educational attainment‡	0.104	0.019 – 0.189	0.02	-0.011	-0.024 – -0.001	0.08
Presence of Plaques, Yes	0.560	0.091 – 1.029	0.02	0.011	-0.058 – 0.081	0.75

Values represent Betas and 95% Confidence Intervals adjusted for time between second and third salivary cortisol measurement. †Values represent unadjusted Betas and unadjusted 95% Confidence Intervals. A Beta (β) denotes the change of Area Under the Curve (nmol/L) or Slope (nmol/L/hour) per unit of covariate. ‡ Educational attainment was analysed a continuous variable, with 1 = primary education, 2 = primary education plus a higher not completed education, 3 = lower vocational education, 4 = lower secondary education, 5 = intermediate vocational education, 6 = general secondary education, 7 = higher vocational education and 8 = university.

DISCUSSION

The present study shows that total cortisol exposure is positively associated with the number of plaques of the carotid arteries in an elderly population. These associations were independent of cardiovascular risk factors and socio-demographic factors. We could not detect a relation between diurnal cortisol decline and atherosclerosis of the carotid arteries.

Some methodological issues of our study need to be discussed before we can interpret the findings. First, this study is cross-sectional and therefore cannot demonstrate the chronology of the observed relationships. Second, studies using saliva sampling to determine the diurnal cortisol pattern rely heavily upon participant adherence to the sampling protocol. Samples taken in the early morning are especially sensitive to deviations from the study protocol, as cortisol levels change rapidly after awakening[23-24]. Probably, noncompliance to the study protocol has also influenced the salivary cortisol concentrations. However, it is unlikely that noncompliance is related to plaque score and will therefore result in random misclassification, which most likely leads to underestimation of the effect. This might explain, why we did not find associations of the slope analyses with atherosclerosis, as the steepness of the slopes depends heavily on the first cortisol measurement. And it could also explain why we did not observe associations with the separate morning cortisol measures in the adjusted models in our study. Third, although we controlled for major confounders like smoking, we cannot rule out residual confounding by unknown factors.

Table 3. The Effects of Different Cortisol Measures on Plaque Score of Atherosclerosis.

		Model 1 Age and sex adjusted		Model 2 Confounder adjusted		Model 3 Confounder and Mediator adjusted	
	N	β (95% CI) *	P-value	β (95% CI) *	P-value	β (95% CI) *	P-value
Separate Cortisol Measures							
$Cort_{aw}$†, 10 nmol/L	1866	0.02 (-0.09 – 0.12)	0.72	0.03 (-0.07 – 0.13)	0.57	0.02 (-0.08 – 0.12)	0.68
$Cort_{aw+30}$‡, 10 nmol/L	1818	0.09 (0.00 – 0.18)	0.05	0.06 (-0.03 – 0.15)	0.20	0.06 (-0.03 – 0.14)	0.22
$Cort_{5pm}$§, 10 nmol/L	1866	0.40 (0.11 – 0.69)	0.006	0.35 (0.07 – 0.64)	0.01	0.35 (0.07 – 0.63)	0.01
$Cort_{bed}$‖, 10 nmol/L	1866	0.51 (0.06 – 0.97)	0.03	0.24 (-0.21 – 0.68)	0.30	0.21 (-0.23 – 0.65)	0.35
Summary Cortisol Measures							
AUC#**, per SD	1818	0.12 (0.04 – 0.20)	0.005	0.08 (0.00 – 0.17)	0.04	0.08 (0.00 – 0.16)	0.04
Slope, per SD	1866	0.01 (-0.07 – 0.09)	0.80	-0.01 (-0.09 – 0.07)	0.88	0.00 (-0.08 – 0.08)	0.98

*Values represent adjusted Betas and adjusted 95% Confidence Intervals. †Salivary cortisol level at awakening. ‡Salivary cortisol level 30 minutes after awakening. §Salivary cortisol level at 5pm. ‖Salivary cortisol level at bedtime. #AUC = Area Under the Curve. Model 1 is adjusted for age, sex and time between awakening and saliva collection in the analyses of the separate cortisol analyses and for age, sex and time between second and third measurement in the analyses of the AUC. Model 2 is adjusted as for model 1 plus current smoking status and month of saliva collection. Model 3 is adjusted as for model 2 plus educational attainment, systolic blood pressure, diabetes mellitus, BMI, total and HDL cholesterol, blood pressure lowering medication and aspirin and statin use. A Beta (β) denotes the change in Plaque Score per unit of cortisol measure. **All analyses with AUC are additionally adjusted for time between second and third salivary cortisol measurement.

Table 4. Relationship between Tertiles of the Area Under the Curve (AUC) and tertiles of Slope and Plaque Score and Presence of Atherosclerotic Plaques.

		Plaque Score		Presence of Atherosclerotic Plaques	
Variable	N	Mean (95% CI)*	P-value	OR (95% CI)†	P-value
AUC#					
Lowest tertile	606	2.80 (2.67 – 2.94)	reference	1 (reference)	-
Middle tertile	606	2.83 (2.69 – 2.96)	0.80	0.96 (0.68 – 1.34)	0.79
Highest tertile	606	3.08 (2.95 – 3.22)	0.005	1.37 (0.94 – 2.00)	0.097
Slope					
Steepest tertile	622	2.89 (2.75 – 3.03)	reference	1 (reference)	-
Middle tertile	622	2.94 (2.80 – 3.07)	0.62	1.20 (0.84 – 1.72)	0.31
Flattest tertile	622	2.88 (2.75 – 3.02)	0.96	0.83 (0.59 – 1.18)	0.30

* Values represent adjusted means with adjusted 95% Confidence Intervals. †Values represent adjusted Odds Ratios and adjusted 95% Confidence Intervals. Analyses are adjusted for age, sex, time between second and third measurement, plus current smoking status, month of saliva collection, educational attainment, systolic blood pressure, diabetes mellitus, BMI, total and HDL cholesterol, blood pressure lowering medication and aspirin and statin use.

To the best of our knowledge, the present study is the first study to evaluate the effects of HPA-axis regulation on atherosclerosis in an elderly population. Previously, only one epidemiological study has examined the effects of HPA-axis regulation and atherosclerosis in adults. This study, performed by Matthews *et al.*[19] was done in a relatively young population, with a mean age of 40 years. Matthews showed that a reduced diurnal cortisol decline, but not total cortisol exposure, was associated with coronary calcification. The prevalence of coronary calcification in this population, however, was only 8.1%. Advantages of our study are the larger sample size and the higher prevalence of atherosclerosis due to an older age of the population: the mean age of our population was 75 years and more than 85% of the participants had one or more plaques (mean plaque score was 2.9). In contrast with the findings of Matthews *et al.*, we found that total cortisol exposure was associated with measures of atherosclerosis and that diurnal cortisol decline was not. Although, our results confirm the hypothesis that total cortisol exposure might be an independent risk for atherosclerosis, the effect estimates were relatively small: persons with AUC values in the highest tertile had a mean increase of 0.3 plaques. We did not expect diurnal cortisol decline not to be related to plaque score. In our study, we found that higher age was associated with flatter slopes. Possibly, in the elderly the effects of cortisol levels are more important in the development of atherosclerosis than diurnal cortisol decline.

Other studies performed in patient groups and healthy volunteers are also in agreement with the notion that cortisol might play a role in the development of atherosclerosis. Eller *et al.*[25-26] found that the level of salivary cortisol 1 hour after awakening and the reactivity in cortisol the first hour after awakening were associated with higher intima media thickness (IMT) of the common carotid arteries in a group of 84 women. Furthermore, they found that the awakening cortisol response was related to progression in IMT in women but not in men[26]. Troxler *et al.*[27] studied 71 male outpatients who underwent coronary angiography because

of medical reasons, as part of their medical evaluation as a United States Air Force aircrew member. They found significant correlations between elevated morning plasma cortisol levels and moderate to severe coronary atherosclerosis. Alevizaki *et al.*[28] showed in a group of 46 subjects undergoing coronary angiography for suspected coronary artery disease, that high morning anticipatory stress cortisol levels predict severity of coronary artery disease. Additionally, Peppa-Patrikiou *et al.*[29] showed that the IMT of the carotid arteries of insulin dependent diabetes mellitus patients is already increased in adolescence. The higher level was positively related to urinary free cortisol.

Several biological mechanisms might explain the association between HPA-axis functioning and atherosclerosis. Cortisol increases glucose levels and is an important factor in the development of diabetes mellitus[18]. Indeed, in our study we found a strong association between total cortisol exposure and diabetes mellitus. However, adjustment for diabetes mellitus did only marginally change our results. Furthermore, previous studies found that dysregulation of the HPA-axis is associated with hypertension[16-17], increased heart rate[30] and total and LDL cholesterol[18]. In the present study, total cortisol exposure was associated with systolic blood pressure. We corrected for systolic blood pressure, total cholesterol and HDL cholesterol in our analyses and found that these factors did not explain the association between total cortisol exposure and number of plaques. Evidence is accumulating that the influence of glucocorticoids on cardiovascular outcome is not mediated exclusively by known cardiovascular risk factors, but may also be the result of direct effects in the blood vessel wall. Inflammation plays a pivotal role in the development of atherosclerosis and cortisol is implicated in the regulation of the immune system[31-32]. Increased local cortisol levels in the blood vessels may promote perivascular inflammation[33]. Furthermore, anti-inflammatory treatment with glucocorticoids may enhance calcification within arteriosclerotic lesions[34]. However, we cannot rule out that higher cortisol levels are a marker of other, pathological, processes, which in themselves promote the development of atherosclerotic lesions.

In summary, we showed that in an elderly population higher total cortisol exposure while awake was associated with number of atherosclerotic plaques of the carotid arteries. This association was independent of cardiovascular risk factors and socio-demographic factors. Our results confirm the hypothesis that total cortisol exposure may be an independent risk factor in the development of atherosclerosis. Prospective studies are needed to confirm the associations.

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Chapter 7

Effect of glucocorticoid receptor gene polymorphisms in Guillain-Barré syndrome

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ABSTRACT

Guillain-Barré syndrome (GBS) is a post-infectious immune-mediated polyneuroradiculopathy, in which host factors influence disease susceptibility and clinical course. Single nucleotide polymorphisms (SNPs) in the glucocorticoid receptor gene (GR) influence the sensitivity to glucocorticoids and are related to both microbial colonization and susceptibility to develop autoimmune disease. This genetic variation may therefore also influence the chance to develop GBS. In this study, we genotyped 318 GBS patients and 210 control subjects for 5 known SNPs in the GR gene. We could distinguish 6 different GR haplotypes of which two carried the *Bcl*I polymorphism: haplotype 1, which consists of the minor allele of *Bcl*I in combination with the common variant of *TthIII*I and haplotype 2, which carries the minor allele of *Bcl*I as well as the minor allele of *TthIII*I. The GR haplotypes were not related to susceptibility to develop GBS. Carriers of haplotype 2 had more frequently preceding diarrhoea, serum antibodies to GM1 and GD1a, and more severe muscle weakness at entry. Haplotype 1 carriers had a significantly better prognosis. In conclusion, GR haplotypes are not a susceptibility factor for GBS. However, haplotypes carrying the minor allele of the *Bcl*I polymorphism were related to the phenotype and outcome of GBS.

INTRODUCTION

Guillain-Barré syndrome (GBS) is an acute polyneuroradiculopathy with a variable clinical phenotype and outcome. GBS is mostly triggered by an aberrant immune response to a preceding infection. *Campylobacter jejuni* is the most frequent antecedent infection in GBS. An important factor in this type of infection is the presence of molecular mimicry with gangliosides, which induces a cross-reactive and neurotoxic immune response to the gangliosides GM1 and GD1a[1-2]. In the majority however, *C. jejuni* infections are not followed by GBS, indicating that genetic host factors may influence the development of such an immune response. Previous studies gave preliminary evidence that host genetic polymorphisms are associated with the occurrence of anti-ganglioside antibodies, as well as the clinical heterogeneity and outcome of GBS[3-4].

Glucocorticoids and sensitivity to glucocorticoids are important regulators of the immune response[5-7]. In the normal population a substantial variability in the sensitivity to glucocorticoids exists, which is partly explained by single nucleotide polymorphisms (SNPs) of the glucocorticoid receptor gene (GR)[8-9]. The ER22/23EK and GR-9*beta* SNPs are related with a decreased glucocorticoid sensitivity, while the *Bcl*I and N363S SNPs are related to an increased glucocorticoid sensitivity[9-10]. Recent studies showed, that these SNPs all influence the chance to develop autoimmune disease[11-12]. In addition, the ER22/23EK polymorphism is associated with an increased and the GR-9*beta* SNP with a decreased susceptibility to Staphylococcus *aureus* nasal carriage[13]. Considering the important role of infections and autoimmune responses in the pathogenesis of GBS, GR polymorphisms may contribute to disease susceptibility and the clinical course in these patients as well.

In the present study, we determined whether GR haplotypes are a susceptibility factor for GBS, or are associated with specific antecedent infections, clinical subgroups or outcome.

MATERIALS AND METHODS

Study population

In this study were included 318 Dutch patients (median age at disease onset 46.0 years, range 4–82 years, male/female ratio = 1.16). All patients fulfilled the diagnostic criteria for GBS[14]. As controls we used 210 healthy Dutch blood bank donors (median age 35 years, range 19–60 years, male/female-ratio = 0.65). The protocol of this study was reviewed and approved by the Medical Ethical Committee of the Erasmus Medical Center, and all patients and healthy controls gave their written informed consent.

Of the patients, 155 participated in one of the Dutch randomised clinical trials, 41 in a national survey on infections and course of disease in mild forms of GBS, 14 in a trial investigating the therapeutic effect of amantidine on fatigue, 71 in observational studies, including one examining the effects of physical activity on fatigue and an ongoing survey studying heterogeneity

in symptoms and course of disease[15-21]. The remaining 37 patients had been admitted to our institutions with GBS but not taken part in other research studies.

Data collection

Detailed demographic, clinical and serological data were available from the participants of the Dutch randomised clinical trials[15-18]. At baseline, information was obtained on symptoms of preceding infections, including presence of diarrhoea within 3 weeks before onset of the first symptoms of GBS. Additionally, pre-treatment blood samples obtained within 2 weeks of onset of weakness, were used to screen for *C. jejuni* serology and the presence of IgM, IgG and IgA antibodies to the gangliosides GM1 and GD1a[22-23]. Sera were all tested in 1/100 dilutions in ELISA using methods and criteria for positivity described previously[23]. During a follow-up of 6 months, disease severity was determined at 17 to 23 standard time points by measuring the GBS disability score and Medical Research Council (MRC) sum score[24-26]. The GBS disability score reflects the functional status of patients with GBS, in which 0 denotes healthy; 1, having minor symptoms and signs but fully capable of manual work; 2 able to walk ≥ 10 meter without assistance; 3, able to walk ≥ 10 meter with a walker or support; 4, bedridden or chair bound (unable to walk 10 meter with a walker or support); 5, requiring assisted ventilation for at least a part of the day; and 6, dead. The MRC sum score indicates the muscle strength of patients, which was generated by the sum of MRC scores from six muscles in the upper and lower limbs on both sides so that the score ranged from 60 (normal) to 0 (quadriplegic). A severe form of disease was defined as an MRC sum score of 40 or less at study entry and a recovery was defined as reaching a GBS disability score of 2 or less (being able to walk independently). MRC sum score at nadir was defined as the lowest MRC sum score during follow up. Overall, 27.4% of the included GBS patients experienced preceding diarrhoea, 26.5% had a serologically confirmed *C. jejuni* infection, 29.4% were severely impaired based on an MRC sum score of ≤ 40 at study entry and 85.9% recovered during follow-up (GBS disability score ≤ 2). 42.6% of the patients had an MRC sum score of ≤ 40 at nadir. Anti-GM1 and anti-GD1a antibodies were present in 24.0% and 8.0% of the patients, respectively. Patients were treated with intravenous immunoglobulin (IVIg) only (47.8%), IVIg and methylprednisolone (44.9%) or plasma exchange (7.3%).

Genotyping

Isolation of genomic DNA from EDTA anti-coagulated peripheral blood samples was performed with the use of the Invisorb® MaxiBlood kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. At the end of the isolation procedure, the DNA samples were dissolved in 0.1 TE buffer (1mM Tris-HCL (pH 7.5) + 0.1 mM EDTA) and stored at -80° C. Polymerase chain reaction amplification and genotyping were performed using 5 ng genomic DNA for the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, California). Primer and probe sequences were optimised using the single nucleotide polymorphism assay-by-design service of Applied Biosystems (for details, see http://store.appliedbiosystems.com). Reactions were

performed with the TaqMan Prism 7900HT sequence detection system (Applied Biosystems) in a 384-well format. To confirm the reproducibility of the genotyping results, 45 of 528 (8.5%) randomly selected samples were genotyped a second time using the same method. No inconsistencies were observed.

All participants were genotyped for 5 known GR gene polymorphisms. Fig. 1 schematically depicts this gene and the location of the 5 polymorphisms and their specific nucleotide variations. More detailed information on location of glucocorticoid receptor gene polymorphisms ER22/23EK (rs6189 and rs6190), N363S (rs6195), *Bcl*I (rs41423247), *TthIII*I (rs10052957) and GR-*9beta* (rs6198) has been described elsewhere[9, 13, 27].

We used the genotype data for each of the 5 polymorphisms to distinguish the haplotypes using the program PHASE, which implements a Bayesian statistical method for reconstructing haplotypes (Fig. 1)[28]. Haplotype 1 is characterized by the G allele of the *Bcl*I polymorphism and the common variant of the *TthIII*I polymorphism, whereas haplotype 2 carries the G allele of the *Bcl*I polymorphism and the T allele of the *TthIII*I polymorphism, which is the minor variant[9]. Haplotype 3 is characterized by the G allele of the *GR-9β* polymorphism in combination with the T allele of the *TthIII*I polymorphism[11]. Haplotype 4 consists of the A allele of the ER22/23EK polymorphism, which is a combination of 2 linked single-nucleotide variations in codons 22 and 23, leading to an arginine-to-lysine change in codon 23 in the transactivation domain, in combination with the G allele of the *GR-9β* polymorphism and the T allele of the *TthIII*I polymorphism[9]. Haplotype 5 is characterized by the presence of the G allele of the N363S polymorphism, which results in an asparagine-to-serine amino acid change. This polymorphism is mutually exclusive with all other minor alleles of the polymorphisms.

Statistical analyses

Hardy-Weinberg Equilibrium (HWE) was calculated according to standard procedures using Chi-square analyses. To analyse the effects of the 5 haplotypes on disease susceptibility and disease characteristics, odds ratios (OR) and 95% confidence intervals (95% CI) adjusted for age and sex were calculated.

Cox regression analyses were performed in patients with a GBS disability score of 3 or higher at entry (N = 183) to determine the effects of GR haplotypes on disease recovery. Survival analyses were adjusted for age and sex (model 1) and additionally for other prognostic factors including GBS disability score at study entry and preceding diarrhoea (model 2). Because data on diarrhoea were missing for 15 patients (8.2%), a missing category was added. The proportional hazards assumption was checked visually with 'log-log' plots. All statistical analyses were performed using SPSS for Windows, release 12.0.1. Results with 2-sided *P* values of <.05 were considered to be statistically significant.

RESULTS

Frequencies of the haplotype alleles are presented in Fig. 1. All GR SNPs were in HWE. Table 1 shows the frequencies of the GR gene haplotypes in healthy controls and in GBS patients. Because of the low numbers of homozygous carriers of GR haplotypes, subjects were analysed as carriers (1 or 2 copies of haplotype allele) versus non-carriers (0 copies of haplotype allele). The GBS patients and healthy controls did not differ with respect to GR haplotype frequencies (Table 1).

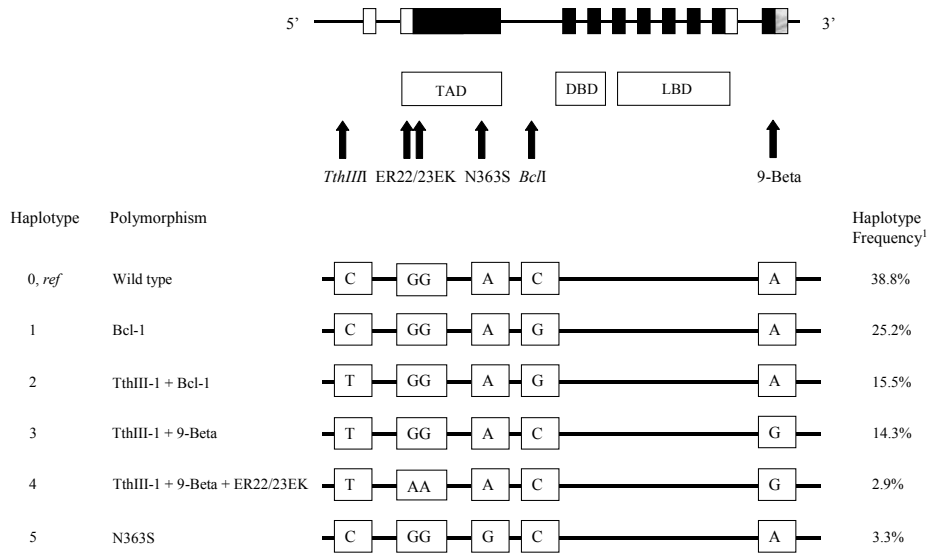


Figure 1 Schematic overview of Glucocorticoid Receptor gene haplotypes.
Haplotypes and haplotype frequencies of the glucocorticoid receptor gene. Nucleic acid changes are indicated. TAD, transactivating domain; DBD, DNA-binding domain; LBD, ligand-binding domain; G, guanidine; A, adenosine; T, thymine and C, cytidine. [1]Haplotype frequencies are based on the results current study in 210 control subjects.

Table 2 presents the associations of haplotype 1 and 2 and disease characteristics of GBS patients. Haplotype 2 carriers, compared to non-carriers, more often had preceding diarrhoea (OR 2.07, 95% CI, 1.06 – 4.06, p = 0.03) and a severe form of disease based on the MRC sum score at study entry (OR 2.60, 95% CI, 1.37 – 4.96, p = 0.004). A similar trend was observed for MRC sum score at nadir, although this relation was not statistically significant (OR 1.50, 95% CI, 0.80 –2.81, p = 0.20). Furthermore, anti-GM1 and anti-GD1a antibodies were more frequently present in carriers of haplotype 2 compared to non-carriers (OR 2.38, 95% CI, 1.11 – 5.11, p = 0.03 and OR 6.00, 95% CI, 1.79 – 20.08, p = 0.004 respectively). In line with this, carriers of haplotype 2 seemed to have more often a positive serology for *C. jejuni* than non-carriers although the difference was not statistically significant (OR 1.89, 95% CI, 0.87 – 4.13, p = 0.11). Post hoc analysis showed, that carriers of the *Bcl*I polymorphism had a significantly higher chance of having a

Table 1 Glucocorticoid receptor gene haplotypes in healthy controls and in Guillain-Barré syndrome patients.

Haplotype, Copy number	Healthy controls (N = 210)		GBS patients (N = 318)		Odds ratios (95% CI)[1] (carriers vs. non-carriers)[2]	p
Haplotype 1						
0	123	58.6 %	187	58.8 %	*reference*	-
1	68	32.4 %	119	37.4 %	0.88 (0.60 – 1.29)	0.52
2	19	9.0 %	12	3.8 %		
Haplotype 2						
0	151	71.9 %	238	74.8 %	*reference*	-
1	53	25.2 %	72	22.6 %	0.88 (0.58 – 1.34)	0.55
2	6	2.9 %	8	2.5 %		
Haplotype 3						
0	155	73.8 %	231	72.6 %	*reference*	-
1	50	23.8 %	82	25.8 %	1.00 (0.66 – 1.53)	0.99
2	5	2.4 %	5	1.6 %		
Haplotype 4						
0	199	94.8 %	298	93.7 %	*reference*	-
1	10	4.8 %	19	6.0 %	1.01 (0.45 – 2.28)	0.97
2	1	0.5 %	1	0.3 %		
Haplotype 5						
0	196	93.3 %	289	90.9 %	*reference*	-
1	14	6.7 %	29	9.1 %	1.38 (0.67 – 2.82)	0.38
2	0	0 %	0	0 %		

[1]Values represent Odds ratios, 95% Confidence Intervals and p-values adjusted for age and sex.
[2]Heterozygous and homozygous carriers of GR haplotypes were combined in the analyses.

positive serology for *C. jejuni* (OR 2.37, 95% CI, 1.11 – 5.08, p = 0.03). No significant association was found between the other GR haplotypes and clinical characteristics of GBS patients.

To summarize, the GR haplotype 2 in GBS is related with the subgroup of patients with low MRC sum score at study entry, preceding diarrhoea, and presence of serum anti-GM1 and anti-GD1a antibodies. As these features are all related, we performed a multivariate logistic regression analyses for these 4 disease characteristics in which we adjusted for the other 3 associated factors. After these additional adjustments, only the associations between haplotype 2 MRC sum score at entry and anti-GD1a antibodies remained statistically significant (OR 2.08, 95% CI 1.02 – 4.21, p = 0.04 and 4.06, 95% CI 1.08 – 15.29, p = 0.04).

The effects of GR haplotypes on disease recovery were studied by Cox regression analysis. During the follow-up period, 158 (85.9%) participants reached independent walking (GBS disability score ≤ 2). The hazard ratios (HR) and 95% CI for this event for haplotypes 1 and 2 are given in Table 3. Heterozygous and homozygous carriers of haplotype 1 had a significantly higher chance of being able to walk independently compared to non-carriers adjusted for age and sex (model 1: HR 1.42, 95% CI, 1.02 – 1.98, p = 0.04 and HR 2.21, 95% CI, 1.01 – 4.85, p = 0.05 respectively). Additional adjustment for main prognostic factors including GBS disability score at study entry and preceding diarrhoea (model 2) and methylprednisolone (data not shown) changed the results only marginally. As a dose allele effect was present, we additionally

Table 2 Haplotype 1 and haplotype 2 of glucocorticoid receptor gene in relation to clinical characteristics of Guillain-Barré syndrome patients.

	Haplotype 1[1]				Haplotype 2[1]			
	Non-carriers	Carriers	OR (95% CI)[2]	p	Non-carriers	Carriers	OR (95% CI)[2]	p
Antecedent infection								
Diarrhoea (N = 197)	34/120 (28.3%)	20/77 (26.0%)	0.88 (0.46 – 1.70)	0.71	33/142 (23.2%)	21/55 (38.2%)	2.07 (1.06 – 4.06)	0.03
C. jejuni infection[3] (N = 166)	22/100 (22.0%)	22/66 (33.3%)	1.76 (0.88 – 3.56)	0.11	30/127 (23.6%)	14/39 (35.9%)	1.89 (0.87 – 4.13)	0.11
Anti-ganglioside antibodies								
GM1 (N = 175)	24/105 (22.9%)	18/70 (25.7%)	1.22 (0.60 – 2.50)	0.58	26/131 (19.8%)	16/44 (36.4%)	2.38 (1.11 – 5.11)	0.03
GD1a (N = 175)	9/105 (8.6%)	5/70 (7.1%)	0.74 (0.23 – 2.37)	0.61	6/131 (4.6%)	8/44 (18.2%)	6.00 (1.79 – 20.08)	0.004
MRC sum score ≤ 40								
At study entry (N = 214)	36/132 (27.3%)	25/82 (30.5%)	1.17 (0.64 – 2.16)	0.61	36/157 (22.9%)	25/57 (43.9%)	2.60 (1.37 – 4.96)	0.004
At nadir (N = 209)	57/131 (43.5%)	32/78 (41.0%)	0.88 (0.50 – 1.57)	0.67	62/155 (40.0%)	27/54 (50%)	1.50 (0.80 – 2.81)	0.20

[1]Heterozygous and homozygous carriers of haplotypes were combined in the analyses.

[2]Values represent Odds Ratios, 95% Confidence Intervals and p-values adjusted for age and sex.

[3]*C. jejuni* infection was defined by standard serological methods.

Table 3 Hazard Ratios and 95% CI of being able to walk independently for haplotypes 1 and haplotype 2 of the glucocorticoid receptor gene.

Haplotype, Copy number	N	Model 1[1]		Model 2[2]	
		HR (95% CI)[3]	p	HR (95% CI)[3]	p
Haplotype 1					
0	113	*reference*	-	*reference*	-
1	63	1.42 (1.02 – 1.98)	0.04	1.52 (1.09 – 2.13)	0.02
2	7	2.21 (1.01 – 4.85)	0.05	2.16 (0.98 – 4.75)	0.06
Haplotype 2					
0	137	*reference*	-	*reference*	-
1	42	0.80 (0.55 – 1.17)	0.25	0.85 (0.58 – 1.26)	0.42
2	4	0.40 (0.10 – 1.64)	0.21	0.44 (0.11 – 1.81)	0.26

[1]Model 1 is adjusted for age and sex.
[2]Model 2 is adjusted sex and for main prognostic factors including age, GBS disability score at entry and preceding diarrhoea.
[3]Values represent Hazard Ratios and 95% Confidence Intervals.

performed trend analyses. In a fully adjusted model (model 2), per one haplotype 1 allele increase, the HR of being able to walk independently again was 1.50 (95% CI, 1.14 – 1.98, p = 0.004). Fig. 2 shows the cumulative chance of 'recovery' (reaching an GBS disability score of 2 or less) for homozygous, heterozygous and non-carriers of haplotype 1. There were no significant associations between other GR haplotypes and disease recovery.

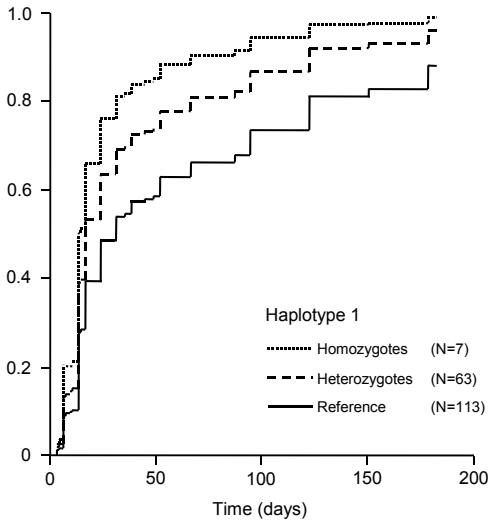


Figure 2 Proportion of patients in time being able to walk independently (GBS disability score ≤ 2) for haplotype 1 of glucocorticoid receptor gene. Survival curves are adjusted for sex and main prognostic factors including age, GBS disability score at entry and preceding diarrhea.

DISCUSSION

This study shows that gene polymorphisms in the GR affecting glucocorticoid sensitivity are not a susceptibility factor to develop GBS. However, haplotype 2 was associated with a severe subform of GBS based on an MRC sum score ≤ 40 at study entry, preceding diarrhoea and the presence of serum anti-GM1/GD1a antibodies. Haplotype 1 carriers more frequently had a good recovery, irrespective of the main prognostic factors and the type of treatment received. Both haplotypes are defined by the *Bcl*I polymorphism of the GR gene, which has been associated with an increased sensitivity for glucocorticoids previously. These findings indicate that GR gene polymorphisms may be associated with the clinical phenotype and recovery of GBS.

In this study we found significant associations with haplotype 1 and haplotype 2. Both haplotypes carry the minor variant of the *Bcl*I polymorphism. Van Rossum *et al.* showed that carriers of this polymorphism have a larger suppression of cortisol secretion after 0.25 mg dexamethasone[29]. This association was observed in an allele dosage way and suggests an increased sensitivity to glucocorticoids *in vivo*. Nonetheless, the molecular mechanism of the *Bcl*I polymorphism, has not been clarified. This intronic polymorphism consists of a C to G mutation, 646 nucleotides downstream from exon 2[27]. Intronic variations may influence the splicing process. However, the *Bcl*I polymorphism is not located near a regulatory splice site. Possibly, *Bcl*I is not functional on itself, but is linked to another polymorphism in the promotor region of the GR gene, which could result in increased GR expression or a variant 3'-untranslated region, which could increase stability of mRNA.

Most previous studies with GR SNPs have analysed the *Bcl*I polymorphism independently and did not take linkage of this SNP with other possible functional polymorphisms into account. However, two studies examined the GR gene in more detail and used tagging SNPs to analyse the effect of common genetic variation on glucocorticoid sensitivity [30-31]. Comparable to our study, both studies described four common GR haplotypes, with a frequency > 5%, of which two haplotypes carry the minor *Bcl*I variant [31]. Strikingly, only one of the two haplotypes carrying *Bcl*I was associated with increased sensitivity to glucocorticoids[30-31]. By comparing haplotype frequencies and using information on linkage disequilibrium of GR SNPs available in the HapMap database (HapMap data release no. 23), we could infer that this functional haplotype corresponds to haplotype 2 in our study and that the other *Bcl*I haplotype matches haplotype 1.

By what mechanism can these GR gene SNPs influence the clinical phenotype and outcome in GBS? Cortisol has an inhibitory effect on both innate and acquired immune function[5-7]. The GR haplotypes determine the sensitivity to glucocorticoids, which influences the effects of cortisol on endothelial cells and immune competent cells, including T-cells, B-cells, neutrophils and macrophages, which may all be important in the pathogenesis of GBS. Most of the effects of cortisol on the immune system are studied in patients taking exogenous glucocorticoids. Well known effects are inhibition of chemotaxis and bactericidal activity in neutrophils and

monocytes, lymphopenia, decreased macrophage function and disturbed complement function[32]. However, acutely, glucocorticoid treatment may promote B cell antibody secretion indirectly via inhibition of suppressor T cell function[33]. Some anti-ganglioside antibodies are neurotoxic and play an important role in the pathogenesis of GBS. It could be that persons carrying a haplotype with an increased sensitivity to cortisol may have a more pronounced antibody secretion following an infection, leading to a more severe disease course. In addition to this, glucocorticoids have profound effects on phagocytic cells and endothelial cells, which may lead to different susceptibility to specific infections[32]. Furthermore, glucocorticoids may have neuroprotective effects and this may result in improved axonal outgrowth after nerve damage[34-35].

Glucocorticoids in general are not effective in the treatment of GBS. Although they may have an additional short term effect when added to IVIg treatment[36]. Polymorphisms in GR gene might influence the response to glucocorticoid treatment in patients with immune mediated disease. In our study, however, we were not able to detect a subgroup of patients who benefitted more by treatment with methylprednisolone (data not shown). Our study probably was underpowered to detect methylprednisolone-haplotype interactions, as data on recovery was available for only 85 participants who received additional methylprednisolone treatment. In addition, the effect of these GR polymorphisms in the specific situation of 500 mg methylprednisolone treatment for five days, is unknown. Disregarding treatment, our study indicates that persons carrying haplotype 1 overall have a better prognosis, even after adjustment for main prognostic factors.

Some methodological issues of our study can be raised. First, in our study more than 90% of GBS patients had a GBS disability score of > 3 at study entry, indicating that severe GBS was overrepresented. This may limit the generalization of our findings, especially to the ~30% of patients with a mild form of GBS. Second, our findings are based on a population of Dutch GBS patients, which may differ from patients from other geographical areas with respect to genetic or environmental backgrounds. Third, genetic association studies require large number of patients. We used the largest cohort of GBS patients included thus far, but the number of patients is probably insufficient to detect associations for the infrequent GR haplotypes 4 and 5. This may also explain why there is no association with haplotype 3 carrying the GR-9*beta* SNP, which follows a recessive model and more homozygotes are needed to detect associations[13, 37]. Furthermore, information on clinical characteristics and outcome was not available for all patients (ranging from n = 166, 47.8% for *C. jejuni* serology of GBS and n = 214, 67.3% for MRC sum score at study entry). This may have led to bias towards finding no significant associations with *C. jejuni* serology. In the present study, haplotype 2 carriers more often have a severe form of GBS based on the MRC sum score at study entry. However, this association was not significant at nadir. Possibly, other factors that influence the disease course, including treatment, may have interfered with the effect of this GR haplotype 2. On the other hand, our positive findings should also be interpreted with caution.

In conclusion, our study shows that haplotypes affecting sensitivity to glucocorticoids are not a general susceptibility factor for GBS. Subgroup analysis showed that haplotype 2 is associated with a more severe subtype of GBS and that carriers of haplotype 1 have a better prognosis on the GBS functional disability score. Future studies are needed to replicate these findings and to elucidate the role of glucocorticoids in the clinical aspects of GBS.

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Chapter 8

General Discussion



8.1 RATIONALE

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) regulates tissue specific cortisol concentrations by converting inert cortisone into active cortisol. Cortisol exerts its function in nearly all tissues and small (tissue-specific) differences in cortisol sensitivity may have profound effects on a susceptibility to disease and metabolic parameters[1]. The studies described in this thesis aimed to explore the role of 11β-HSD1 in hypothalamic-pituitary-adrenal (HPA) axis regulation and features of the metabolic syndrome (MetS). Furthermore, we evaluated the effect of free cortisol, as measured by salivary cortisol concentrations, on atherosclerosis in an elderly population.

8.2 11β-HSD1 AND CORTISONE REDUCTASE DEFICIENCY

Cortisone reductase deficiency (CRD) is a rare condition in which peripheral conversion of inert cortisone into active cortisol does not take place. As a consequence, there is an increased clearance of cortisol, and decreased negative feedback of the HPA axis, which in turn leads to ACTH-mediated adrenal androgen excess. Male patients manifest early in life precocious pseudopuberty and females present with hirsutism, acne, menstrual irregularities and infertility problems. CRD is diagnosed through assessment of urinary cortisol and cortisone metabolites by measuring the tetrahydrocortisol plus 5α-THF/tetrahydrocortisone ratio, which is usually lower than 0.1 in patients (reference range 0.7-1.2)[2].

In 2003, Draper *et al.* proposed a triallelic digenic model of inheritance for CRD, in which three distinct alleles, from two loci (*HSD11B1* and *H6PD*) were necessary for trait manifestation[3]. Two of those alleles occurred relatively frequent in the general population: R453Q (rs688832) is a missense mutation in *H6PD* and has a minor allele frequency of 20% and *HSD11B1* 83,557insA (rs45487298), which is in full LD with 83,597T>G (rs12086634) and has an allele frequency of 14% in the general population. The combined genotype of *H6PD* R453Q homozygosity and *HSD11B1* 83,557insA was predicted to occur in approximately 1% of the general population which seemed not inconsistent if the disease was poorly diagnosed. White attempted to confirm the results of Draper *et al.* by detecting additional CRD patients through genetic screening of 3553 individuals from the Dallas Heart Study, a large population based sample. He found that the minor alleles at each locus occurred much more frequently than previously reported. As a consequence 7.0% of the subjects had the 'CRD genotype', which is inconsistent with the rarity of CRD. Moreover, he found no difference in urinary cortisone to cortisol metabolites between the genotype groups[4].

Our findings in the Rotterdam Study and the FOM study are in agreement with the findings of White. Approximately 4% of the participants presented with the ´CRD genotype´. Persons with the 'CRD genotype' had no elevated adrenal adrogens. In women, however, there was a

trend towards higher androstenedione levels for *HSD11B1* 83,557insA carriers, but the observed differences were much lower than expected for CRD patients. Moreover, we did not detect any significant effects of the CRD genotype or separate polymorphisms on blood pressure, glucose metabolism or anthropometric parameters. The findings of White and the results of our study indicated that it is unlikely that *H6PD* R453Q and *HSD11B1* 83,557insA interact to cause CRD.

The conclusive answer for the genetic background of CRD came from the study of Lavery *et al.*[2]. In a new case with CRD a novel homozygous mutation of *H6PD* was identified. This finding prompted reinvestigation of the cases included in the initial report of Draper *et al.*[3]. It revealed four novel and one previously reported variants of *H6PD* that were not detected in 120 control chromosomes. Expression and activity assays demonstrated loss of function for all reported *H6PD* mutations. The oxo-reductase function of 11β-HSD1 depends on the availability of its co-factor NADPH. *H6PD* is probably the only source of NADPH within the lumen of the ER. Interestingly, heterozygosity for *H6PD* inactivation did not lead to loss of 11β-HSD1 oxo-reductase function[2].

Recently, Lawson *et al*. identified two novel heterozygous mutations in *HSD11B1,* K187N and R137C, in two patients presenting with a milder form of CRD in which *H6PD* was normal. These heterozygous mutations were found to have a dominant negative effect on the formation of functional dimers and explained the genetic cause of CRD in these patients[5].

8.3 11β-HSD1 AND HPA AXIS FUNCTIONING

8.3.1 11β-HSD1 expression at the negative feedback sites of the HPA axis

There is considerable evidence that 11β-HSD1 is important in rodent HPA axis regulation. 11β-HSD1 is widely expressed throughout the rodent brain, including the main negative feedback and regulatory sites of cortisol: the PVN, anterior pituitary, and hippocampus[6-8]. Furthermore, studies with 11β-HSD1 null mice showed that 11β-HSD1 deficiency alters HPA axis function[9-10]. Data on the role of 11β-HSD1 in human HPA axis regulation are scarce.

In this thesis, we studied the role of 11β-HSD1 in human HPA axis functioning in two different ways. First, we examined the expression of 11β-HSD1 at the main negative feedback sites of the HPA axis using human post-mortem autopsy samples. Next, we investigated the effects of common genetic variation in *HSD11B1* on measures of HPA axis activity (see discussion 8.3.2). Both studies suggest that 11β-HSD1 can influence human HPA axis activity. 11β-HSD1 was expressed in many nuclei of the hypothalamus, most prominently in the PVN and the supraoptic nucleus (SON). Moreover, in the PVN, which is one of the main negative feedback sites of the HPA axis, 11β-HSD1 is expressed in the CRH-, as well as AVP-, and OXT- secreting cells. Although all these cell may modulate HPA function, the neurosecretory cells that secret both CRH and AVP are the most important cells of the PVN for PA axis regulation[11-15]. CRH and AVP synergistically stimulate ACTH release from the pituitary corticotrophs. Regeneration of cortisol by 11β-HSD1

may down-regulate CRH and AVP synthesis and secretion, which in turn leads to deactivation of the HPA axis. A limitation of our study was however, that the concentration of 11β-HSD1 antibodies needed to get a good immunofluorescence signal in the colocalization studies of the PVN was high (1:50) and therefore background staining may have biased our results. So, studies using other techniques, for instance mRNA in situ hybridization and qPCR are needed to replicate these findings.

Our study does not provide definitive information concerning the potential role of 11β-HSD1 at the pituitary level. In the anterior pituitary, 11β-HSD1 was expressed in the hormone producing cells as well as folliculostellate cells. However, we were unable to visualize an 11β-HSD1 signal using fluorescence immunocytochemistry. Previously, Korbonits *et al.* showed that 11β-HSD1 was expressed in the FS and growth hormone secreting cells but not in the corticotrophs. So taken the results of this study into account it seems less likely that 11β-HSD1 modulates HPA axis function at the level of the pituitary. However, at this stage the results of our study are non-conclusive.

8.3.2 *HSD11B1* and HPA axis activity

We evaluated the effects of common genetic variation in *HSD11B1* on HPA axis activity using a tagging SNP approach. For this study, we selected 12 SNPs including 11 tagging SNPs. One of those SNPs, rs11119328, was significantly associated with higher salivary cortisol levels at bedtime in all participants and with higher androstenedione levels in postmenopausal women. Two other polymorphisms that are in strong LD with this variant were also associated with higher cortisol levels, but the associations with androstenedione did not reach statistical significance. The combination of increased cortisol as well as higher adrenal androgen levels indicates that rs11119328 carriers have an altered HPA axis set point. We hypothesized that rs11119328 is, or tags an inactivating variant, which leads to lower expression of 11β-HSD1 at the central feedback sites of the HPA axis, resulting in diminished negative feedback. This variant was also associated with an increased risk of incident depressive disorder. This is an interesting finding since 80% of depressed patients have an impaired negative feedback of the HPA axis[16].

A major limitation of our study was that we did not replicate our findings. Non-replication is a major issue in genetic association studies and often reflects false positives in the original reports[17-20]. One explanation for non-replication is multiple testing. Modern genetic association studies typically involve multiple genetic markers and outcomes. In our study we tried to correct for multiple testing by using permutation analyses. Furthermore, we also addressed the problem of multiple testing by analysing different measures of HPA axis activity. However, replication studies are the gold standard and future studies in different study populations are needed to confirm our results.

Another issue is that tagging SNPs may reveal promising signals, but identification of the underlying causal variants often remains elusive. With respect to our gene of interest: little is

known about the functional effects of polymorphisms in *HSD11B1*. Draper *et al.* showed that in vitro, the transcriptional activity of *HSD11B1* constructs containing the intron 4 83,557insA (rs45487298) and 83,597T>G (rs12086634) mutations was 2,5 times lower than constructs containing the wild type variant. Rs11119328 is in strong LD with those variants ($r^2 = 0.75$). However, our strongest associations were observed for rs11119328 and not rs45487298, suggesting that our signal was not caused by rs45487298. Future studies should not only aim to identify the underlying causal variant, but should also include in vitro functionality of this gene variant.

In our study, common genetic variation in *HSD11B1* was associated with salivary cortisol levels at bedtime, but not with the cortisol samples taken in the morning. This is surprising given the fact that the heritability of the cortisol awakening response is larger than that of cortisol levels at the diurnal nadir[21]. Several possible explanations for this discrepancy exist. First, in the view of the complexity of the HPA system, genetic factors that influence cortisol levels at its nadir need not necessarily also impact the cortisol awakening response. Another, possibly more likely, explanation is that studies using saliva sampling to determine the diurnal cortisol pattern rely heavily upon participant compliance.

Kudielka *et al.*[22] showed in a study using electronic monitoring devices that a significant number of participants (26%) did not obtain saliva samples reliably in an ambulatory setting. Furthermore, these authors showed that researchers cannot rely on participants' self-reported sampling times. Subjects who were unaware that their sampling times were measured by electronic monitoring devices indicated that they deviated from the protocol time by a total of 68 ± 8 minutes. Actual deviation based on the electronic monitoring data was twice that. The most important effect of compliance on the cortisol measurement was seen for the cortisol awakening response. Compliant subjects showed a robust increase in cortisol values 30 minutes after awakening, whereas participants who failed to obtain this sample at the correct timing only showed minimal changes from baseline. This indicates that deviation from the study protocol can partially invalidate the cortisol results and mask potential differences between subject groups of interest. This is especially true for samples taken early in the morning[22-23]. Noncompliance with the study protocol probably influenced the salivary cortisol concentrations in our study. This might explain the observed absent relationships between *HSD11B1* SNPs and morning cortisol levels. The influence of variation in sampling time is much smaller for evening cortisol levels, because these levels are much lower and more stable.

8.4 11β-HSD1 AND DEPRESSION

Few authors have previously evaluated the possible role of 11β-HSD1 in depression. Some authors evaluated the ratio of urinary cortisone to cortisol metabolites as a marker for overall 11β-HSD1 and 11β-HSD2 functioning, but the results of these studies have been conflicting[24-26].

We found that carriers of rs11119328, a tagging SNP in *HSD11B1* that was associated with increased HPA axis activity, also had an increased risk of an incident depressive disorder. This finding may provide some insight in the aetiology of depression. A significant percentage of depressed patients have an increased activity of the HPA axis, as shown by elevated basal cortisol levels, as well as an inability to suppress cortisol secretion following administration of exogenous glucocorticoids (GCs)[27-28]. However, it is uncertain if these HPA axis abnormalities are a permanent result of the disease process or if they are an independent risk factor for depression[29].

More recently, genetic epidemiologists have used Mendelian Randomisation to infer causality between biomarkers and disease outcome[30-31]. Polymorphisms associated with higher HPA axis activity produce a natural (Mendelian) randomisation, with individuals allocated to higher and lower HPA axis activation according to a random assortment of alleles during gamete production and fertilization. If increased HPA axis activation would indeed be a risk factor for depression and not merely a result of altered physiology as a result of disease, one expects that in the group with a naturally increased HPA axis activation the incidence of depression is higher.

We showed that subjects carrying an *HSD11B1* polymorphism that was associated with higher measures of HPA axis activity also had in increased risk of incident depression. Can we conclude from this, that higher HPA axis activity is indeed a susceptibility factor for depression? Before we draw this conclusion we need to discuss two limitations of our study. First, the salivary cortisol collection was performed at study wave four of the Rotterdam Study (2002 – 2004), whereas follow-up of depression already started at the second study survey (1993 – 1995). So, theoretically rs11119328 might be associated with an increased risk of depression, which in turn results in (lifelong) increased HPA axis activity. Although this direction of causality seems less likely, the design of our study is inadequate to infer causality. Furthermore, we have not replicated our findings yet. Thus replication studies are needed to confirm our results.

8.5 11β-HSD1 AND METABOLIC SYNDROME

The MetS consists of a cluster of cardiovascular risk factors[32]. The observation that many of these metabolic disturbances are also present in patients with Cushing's syndrome has led to the idea that exposure to excessive GCs is implicated in the pathogenesis of the MetS. However cortisol levels are usually within the normal range and symptoms cannot be fully explained by hyperactivity of the HPA axis alone[33]. Therefore, many authors suggested that increased local generation of cortisol by 11β-HSD1 might be implicated in the MetS[34-36]. This hypothesis got further support after the observation that transgenic mice overexpressing 11β-HSD1 in adipose tissue developed visceral obesity, as well as other features of the MetS, such as insulin resistant diabetes and hyperlipidemia[37]. 11β-HSD1 knockout mice, on the contrary, appeared to be more resistant to hyperglycaemia upon obesity or stress[38].

Several genetic association studies evaluated the effects of *HSD11B1* 83,597T→G (rs12086634), which is in full LD with *HSD11B1* 83,557insA (rs45487298) on traits of the MetS and PCOS[4, 39-41]. These SNPs were interesting candidate SNPs because they were associated with lower 11β-HSD1 transcriptional activity in vitro and initial reports claimed that those SNPs interact to cause CRD[3]. However, this concept is now out of date after the publication Lavery *et al.* in which it was demonstrated that CRD is caused by inactivating mutations in *H6PD* (see discussion 8.2)[2]. Early studies showed that rs12086634 was associated with hypertension and type 2 diabetes in Pima Indians[42-43]. However, we could not replicate these findings in our elderly Caucasian study populations (Rotterdam Study and Frail Old Men Study), nor did we observe any significant effects on other traits of the MetS[44]. These discrepant findings between the different study populations might be explained by racial and age differences, since the effects of polymorphisms may be age-dependent, while they may also exert different effects in the context of different genetic backgrounds[45-46]. Alternatively, the initial findings in Pima Indians were chance findings.

Our next step was to evaluate the effects of other genetic variation in *HSD11B1* using a tagging SNP approach. 11 tagging SNPs were selected using the HapMap database (Public release #20). We related those SNPs to the MetS and traits of the MetS in the Rotterdam Study. Significant findings were replicated in the adult offspring of the population based 1966 Northern Finland Birth Cohort (1966 NFBC). Taking multiple testing into account using permutation analyses only one significant associations was observed: in the Rotterdam Study, rs17317033 was significantly associated with a larger waist circumference in women. A similar trend was observed in 1966 NFBC subjects, however this study was underpowered to detect any significant effects. Further studies are needed to confirm this association. Although central obesity is thought to be one of the key features of MetS, rs17317033 was not associated with any of the other MetS components such as lipid profile, blood pressure and glucose metabolism. This is surprising given the fact that obesity is associated with adverse metabolic consequences such as diabetes and dyslipidemia and that the best predictor of these morbidities is not the total body adipose mass, but the specific quantity of visceral fat[37, 47-48].

We did not detect any effects of rs11119328 on any of the MetS traits. This SNP was associated with higher HPA axis activity and an increased risk of incident depression in the Rotterdam Study (see Chapter 5 and discussion 8.3.2 and 8.4). We hypothesized that rs11119328 is, or tags, an inactivating variant, which leads to 11β-HSD1 activity at the central feedback sites of the HPA axis, resulting in diminished negative feedback. Possibly, increased HPA axis activity compensates for an attenuated generation of cortisol at the tissue level. Alternatively, the detected associations with higher HPA axis measures are false-positive associations.

In conclusion, despite a strong biological hypothesis, we did not find convincing evidence that common genetic variation in *HSD11B1* contributes to susceptibility to the MetS. Can we conclude from this that pre-receptor regulation of cortisol by 11β-HSD1 is not involved in the pathogenesis of the MetS? Although candidate gene studies may provide useful insights in

the actions of a protein of interest, one cannot conclude from negative association studies alone that 11β-HSD1 is not implicated in MetS. It is possible that common genetic variation in *HSD11B1* does not result in functional variants with important biological consequences. Until now, over 500 genetic variants have been identified in the *HSD11B1* gene region, but only 7 polymorphisms are located in the translated regions of *HSD11B1* (db SNP database, build 132). These variants can lead to alterations in the amino acid sequence or introduce a premature stop codon and are therefore potentially functional. However, 5 of the 7 polymorphisms that are located in *HSD11B1* are synonymous SNPs, which do not cause amino acid changes. Two other mutations include a missense mutation in exon 2 (rs111629669) resulting in a threonine to isoleucine change and a frame shift mutation in exon 7 (rs34727634). The minor allele frequencies of both variants are unknown in the db SNP database, which usually means that they are (extremely) rare mutations. In comparison, in the GC receptor gene (GR) over 1600 genetic variants have been identified, of which 40 are located in the coding region including one nonsense and 18 missense mutations. Another issue we need to bear in mind is that we used a minor allele frequency cut off-of 5%. This means that with our approach we did not tag rare genetic variation.

In summary, we did not find convincing evidence that common genetic variation in 11β-HSD1 is important in the pathogenesis of MetS. This does not necessarily mean that 11β-HSD1 is no longer an interesting therapeutic target in patients with the MetS. This is illustrated by a recent study of Rosenstock *et al.,* who showed that when the 11β-HSD1 inhibitor INCB13739 was added to metformin monotherapy in type 2 diabetes patients exhibiting inadequate glycemic control, significant reductions of fasting glucose and HbA1C levels were achieved compared to placebo group. Moreover, this drug was well tolerated, body weight was decreased in the treated group, and in hyperlipidemic patients total cholesterol, low-density lipoprotein cholesterol, and triglycerides were all significantly reduced[49]. Future studies are needed to confirm the efficacy and safety of INCB13739 in the treatment of type 2 diabetes and evaluate its effects on other MetS traits.

8.6 SALIVARY CORTISOL AND ATHEROSCLEROSIS

For several decades the role of stress in the pathogenesis of cardiovascular disease has been debated. One of the possible mechanisms through which exposure to stress might influence the risk of ischaemic heart disease is dysregulation of the HPA axis. We evaluated the associations of two summary measures of HPA axis function on carotid plaques in the Rotterdam Study, a population-based study in the elderly. We found that diurnal cortisol exposure while awake, as measured by the area under the curve (AUC), was related to a higher plaques score, whereas diurnal cortisol decline did not show a significant association with the number of atherosclerotic plaques[50]. These findings are in line with the observation that Cushing's syndrome

patients have increased measures of atherosclerosis[51-54]. Furthermore, studies with healthy volunteers and patient groups confirm that increased cortisol levels are associated with higher measures of atherosclerosis[55-60]. Until now, only one other large epidemiological study has studied the associations of cortisol levels with atherosclerosis. In contrast to our findings, Matthews *et al.* found that diurnal cortisol decline, but not total cortisol exposure, was associated with presence of any coronary calcification in CARDIA[61]. This is an ongoing prospective cohort study of the natural history of cardiovascular risk development starting in young adulthood. There is no clear explanation for the different results between CARDIA and the Rotterdam Study. Differences in age and prevalence of atherosclerosis might possibly explain the discrepant findings; the mean age of our study population was 75 years and 85% of the participants had one of more plaques, whereas CARDIA participants were 40 years on average, and only 8% had signs of coronary calcification.

Some methodological issues of our study need to be discussed. First, this study is cross-sectional and therefore cannot demonstrate the chronology of the observed relationships. Prospective studies are needed to examine the direction of causality. Second, although we did correct for important confounders such as smoking, we cannot rule out that residual confounding might have biased our results. Furthermore, studies using salivary cortisol rely heavily on participant's adherence to the study protocol[22-23]. Probably, noncompliance has also influenced the salivary cortisol concentrations in our study population. However, it is unlikely that noncompliance is related to plaque score and will therefore result in random misclassification, which most likely leads to underestimation of the effect.

8.7 GLUCOCORTICOID RECEPTOR GENE POLYMORPHISMS AND GUILLAN-BARRÉ SYNDROME

Guillan-Barré syndrome (GBS) is an autoimmune-mediated post-infectious polyneuropathy in which (genetic) host factors influence disease susceptibility and clinical course[62]. SNPs in GR are related to microbial colonization and autoimmune disease[63-65]. Considering the important role of infections and autoimmune responses in the pathogenesis of GBS, we hypothesized that GR polymorphisms may contribute to disease susceptibility and clinical course of GBS. To investigate this hypothesis, we genotyped 318 Dutch GBS patients and 210 healthy controls for 5 extensively studied polymorphisms in the GR and constructed 6 haplotypes (Fig. 1)[66].

The GBS patients and healthy controls did not differ with respect to GR haplotypes. However, haplotype 2 was associated with a severe subform of GBS, preceding diarrhoea and the presence of serum anti-GM1/GD1a antibodies. Haplotype 1 carriers more frequently had a good recovery, irrespective of the main prognostic factors and the type of treatment received[66]. Both haplotypes are defined by the minor allele of *Bcl*I, which has been associated with an increased sensitivity for glucocorticoids in vivo previously[67]. The exact molecular mechanism

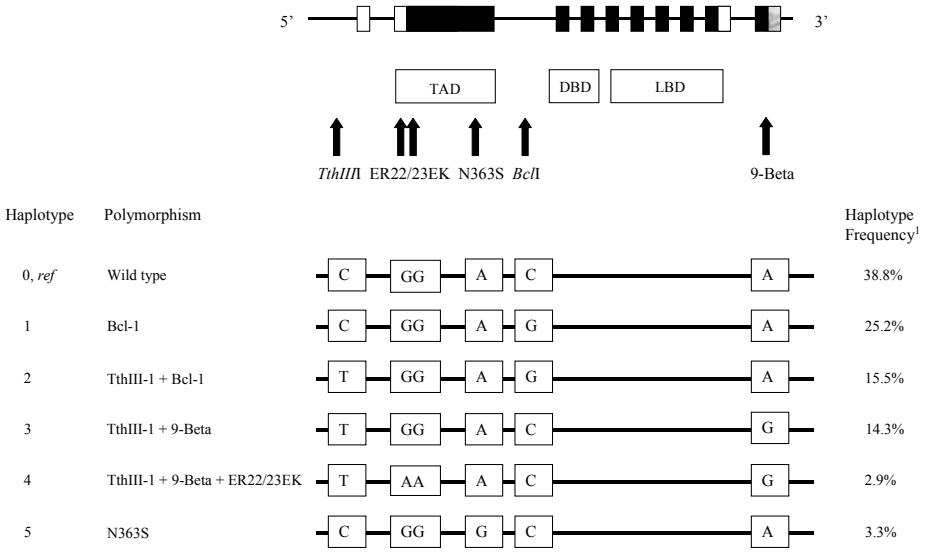


Figure. 1. Schematic overview of Glucocorticoid Receptor gene haplotypes.
Haplotypes and haplotype frequencies of the glucocorticoid receptor gene. Nucleic acid changes are indicated. TAD, transactivating domain; DBD, DNA-binding domain; LBD, ligand-binding domain; G, guanidine; A, adenosine; T, thymine and C, cytidine. [1]Haplotype frequencies are based on the results current study in 210 control subjects.

of this intronic polymorphism has not been clarified[68]. Most previous studies have evaluated the effects of *Bcl*I independently and did not take linkage with other genetic variants into account. However, two previous studies analysed the effect of genetic variation in the GR on GC sensitivity using a tagging SNP approach[69]. In line with our results, both studies described 4 common haplotypes with a frequency > 5%. Two of those haplotypes carried the minor *Bcl*I variant. Interestingly, only the haplotype that corresponded to haplotype 2 in our study was associated with increased sensitivity to GCs. Future studies are needed to further elucidate the functional differences between GR haplotype 1 and 2.

8.8 GENERAL REFLECTIONS ON GENETIC ASSOCIATION STUDIES

In this thesis we used two different strategies to evaluate the effect of common genetic variation in *HSD11B1* (and *H6PD*) on different phenotypes. In Chapter 2, we evaluated the effects of two candidate SNPs, *H6PD* R453Q and *HSD11B1* 83,557insA on different phenotypes related to cortisol metabolism e.g. glucose metabolism, blood pressure and anthropometric measures[44]. In Chapter 4 and Chapter 5, we used a tagging SNP approach to evaluate the effects of common genetic variation in *HSD11B1* on HPA axis activity, depression and traits of the MetS. A major issue of both strategies is a failure to replicate associations. This failure to replicate has led to

scepticism about the validity of genetic association studies. Non-replication is usually a result of a false-positive finding in the initial report, however other possible explanations include false-negative findings in the replication studies and methodological differences between studies such as phenotyping heterogeneity or ethnic differences[17, 45, 70].

Multiple testing, including subgroup analyses and testing of alternative outcome measures, is an important cause of false-positive associations[20, 46]. There are several methods to correct for multiple testing. The traditional Bonferroni correction proposes to lower the threshold for significance to 0.05 divided by the number of tests, but is generally believed to be too conservative[46]. In Chapter 4 and 5 we corrected for multiple testing using permutation analyses, which takes the LD between analyzed genetic markers into account[71]. False-positive associations may also arise with small samples[46]. In a group of 318 GBS patients we found significant associations between two GR haplotypes and clinical characteristics of GBS[66]. Although this is the largest cohort of GBS patient thus far, in the context of genetic association studies this is a very small study sample. Until replication, we should interpret these findings with caution.

After replication, the next steps in genetic association studies are confident localization of the causal variant, confirming functionality with in vitro tests and elucidating the molecular mechanism through which the polymorphism exerts its effect[72]. Identification of the functional genetic variant may be extremely challenging in a tagging SNP setting for several reasons: the complete common SNP set is not known, tagging SNPs may be associated with (functional) distant loci and they may detect more rare variants with a high penetrance[72]. Candidate SNPs are usually chosen on basis of plausible functionality. They are generally located in regulatory elements, result in amino acid changes or previous studies have shown their functional relevance. Many studies have been successful in confirming the in vitro functionality of candidate SNPs, including three GR SNPs: ER22/23EK, GR-9*beta* and N363S[1]. For one of these SNPs, ER22/23EK, the exact molecular mechanism has been unraveled[73].

Choosing candidate SNPs on the basis of prior studies and location in the gene yields the risk of missing functional genetic variation. Therefore, a better strategy to capture all common genetic variation in a gene of interest might be using a tagging SNPs approach. Over the last years, a new hypothesis free approach has become available: the genome-wide association study (GWAS). This method allows examining the effect of all common genomic variation on a trait of interest and may identify new biological pathways. Many GWAS have successfully identified and replicated new loci involved in a variety of complex diseases among others osteoporosis[74], macular degeneration[75], and Alzheimer's disease[76]. GWAS have also detected highly significant hits for (visceral) obesity and other traits of the MetS. While the biological mechanisms of these variants remain unknown, many of the discovered candidates locate near genes that are highly expressed in the brain and hypothalamus, suggesting a role for neuronal control in body weight regulation[77].

8.9 CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, 11β-HSD1 is expressed at the two main negative feedback sites of the HPA axis: the PVN and the anterior pituitary. In the human PVN, 11β-HSD1 is expressed by CRH-, AVP-, and OXT-producing neurons. These observations suggest that hypothalamic production of cortisol by 11β-HSD1 might modulate HPA activity. The role of 11β-HSD1 at the anterior pituitary remained undetermined. We identified one tagging SNP in *HSD11B1* that was associated with higher HPA axis activity and incident depression, confirming that 11β-HSD1 might influence human HPA axis regulation. Another tagging SNP in *HSD11B1* was associated increased waist circumference in women, but not in men. Although increased waist circumference is one of the key features of the MetS, this polymorphism was not associated with other MetS traits. Furthermore, we showed that free cortisol, as measured as salivary cortisol, was associated with atherosclerosis of the carotid arteries in an elderly population.

Future studies are needed to further explore the role of 11β-HSD1 in human HPA axis regulation. First, replication cohorts are needed to validate our observation that rs11119328 is associated with increased measures of HPA axis activation and depression.

Furthermore, exploring 11β-HSD1 expression at the negative feedback sites of the HPA axis in different hyper- and hypocortisolemic disease states may elucidate possible regulatory mechanisms of 11β-HSD1 in HPA axis regulation. Or conversely, reduced 11β-HSD1 expression may contribute to the feedback inhibition in depressed patients.

Although we did not detect any effects of genetic variation in *HSD11B1* on susceptibility to MetS, Rosenstock *et al.* recently showed that adding the 11β-HSD1 inhibitor INCB13739 to metformin monotherapy was successful in lowering fasting glucose and HbA1C levels in patients with type 2 diabetes exhibiting inadequate glycemic control. These findings will probably initiate further research for other indications of this drug. A role for 11β-HSD1 inhibition has also been suggested for a number of conditions including the other components the MetS, cognitive decline, osteoporosis, and atherosclerosis[36, 78-80].

Studies using salivary cortisol in an ambulatory population-based setting are challenging because they rely on participants' compliance. This may be particularly true in elderly populations where at least a number of the subjects suffer from cognitive decline. At present, cheap monitoring devices that can be used on a large scale are not available. Development of those devices will largely improve the quality of cortisol data collection and may improve reproducibility of (genetic) association studies.

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Chapter 9

Summary



SUMMARY

The general aims of this thesis are to evaluate the effects of free cortisol, as measured by salivary cortisol, and the effects of pre-receptor regulation of cortisol by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) on hypothalamic-pituitary-adrenal (HPA) axis regulation and metabolism.

As described in **Chapter 1**, cortisol exerts its function in nearly all tissues. Cortisol levels are regulated by the HPA axis. Local, tissue specific cortisol levels are regulated by two enzymes; 11β-HSD1 and 11β-HSD2. In vivo, 11β-HSD1 converts inert cortisone to active cortisol whereas 11β-HSD2 converts cortisol to cortisone. The oxo-reductase function of 11β-HSD1 depends on the availability of its co-factor NADPH, which is generated by hexose-6-phosphate dehydrogenase (H6PDH).

Cortisone reductase deficiency (CRD) is a rare condition in which peripheral conversion of cortisone into cortisol does not take place. Previously, it was proposed that a combination of *HSD11B1* 83,557insA and *H6PD* R453Q polymorphisms interacts to cause CRD, when at least three alleles are affected. However, in **Chapter 2** we show that a considerable number of subjects in two elderly populations carry the 'CRD genotype' (3.8% in FOM and 4.0% in the Rotterdam Study), which is not consistent with the rarity of CRD. Moreover, we detected no effect of the 'CRD genotype' or the separate polymorphisms on glucose metabolism, body composition, blood pressure, adrenal androgens and dementia.

In **Chapter 3** we studied 11β-HSD1 expression at the two main negative feedback sites of the HPA axis: the anterior pituitary and hypothalamus. 11β-HSD1 is expressed in many hypothalamic nuclei, most prominently in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON). In the PVN, 11β-HSD1 appears to colocalize with vasopressin- (AVP), oxytocin- (OXT) and corticotropin-releasing hormone- (CRH) immunoreactive neurons. These results suggest that hypothalamic production of cortisol by 11β-HSD1 may modulate HPA axis function. In the anterior pituitary, 11β-HSD1 is expressed in the hormone producing cells as well as folliculostellate cells. We could not determine colocalization of 11β-HSD1 in the anterior pituitary, because we were unable to visualize an 11β-HSD1 signal using fluorescence immunocytochemistry in the pituitary.

In **Chapter 4** we evaluate the effects of common genetic variation in *HSD11B1* on HPA axis activity using a tagging SNP approach. For this study, we selected 12 SNPs including 11 tagging SNPs. One of those SNPs, rs11119328, was significantly associated with higher salivary cortisol levels at bedtime in all participants and with higher androstenedione levels in postmenopausal women indicating a higher HPA axis set point. Carriers of this polymorphism also had an

increased risk of incident depression. This suggests a causal relation between HPA axis activation and depression.

It was proposed that local generation of cortisol by 11β-HSD1 in omental fat tissue plays an important role in the aetiology of the metabolic syndrome (MetS). In **Chapter 5** we evaluate the effect of common genetic variation in *HSD11B1* on (traits of) the MetS in two Caucasian study populations. In the Rotterdam Study rs17317033 was significantly associated with a larger waist circumference in women. A similar trend was observed in the 1966 Northern Finland Birth Cohort. However, this study was underpowered to detect any significant effects. This SNP was not related to any of the other MetS traits. None of the selected tagging SNPs was associated with an increased susceptibility to MetS.

Dysregulation of the HPA axis has been suggested as an independent risk factor for ischemic heart disease. We describe in **Chapter 6** that total cortisol exposure while awake is associated with an increased number of atherosclerotic plaques of the carotid arteries in an elderly population. Diurnal cortisol decline was not related to plaque score. Future prospective studies are needed to determine the chronology of the observed relationship.

In **Chapter 7**, the addendum, we showed that glucocorticoid receptor (GR) haplotypes do not contribute to susceptibility to Guillain-Barré syndrome (GBS). However, haplotype 1, which consists of the minor allele of *Bcl*I in combination with the common variant of *TthIII*I and haplotype 2, which carries the minor allele of *Bcl*I as well as the minor allele of *TthIII*I were related to clinical course and outcome of GBS. Although we used the largest cohort of GBS patients thus far, in the context of genetic association studies the number of included subjects was small (n = 318) and until replication, we should interpret these findings with caution.

Chapter 8 contains a general discussion and puts the findings described in this thesis in a broader perspective. Special attention is paid to the strengths and limitations of genetic association studies.

SAMENVATTING

Het doel van dit proefschrift is om de effecten van lokale regeneratie van cortisol door 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) op hypothalamus-hypofyse-bijnier-as (HPA-as) regulatie en metabolisme te bestuderen. Daarnaast hebben wij de associaties van vrij cortisol, gemeten als speeksel cortisol, met atherosclerose bestudeerd.

In **Hoofdstuk 1** beschrijven wij dat cortisol in bijna alle weefsels een belangrijke rol speelt. Cortisolspiegels staan onder controle van de HPA-as. Lokale, weefselspecifieke weefselconcentraties worden gereguleerd door twee enzymen: 11β-HSD1 en 11β-HSD2. In vivo zet 11β-HSD1 inactief cortison om in actief cortisol, waar 11β-HSD2 juist cortisol inactiveert tot cortison. De oxoreductase functie van 11β-HSD1 is afhankelijk van de aanwezigheid van de cofactor NADPH, die wordt gegenereerd door hexose-6-phosphate dehydrogenase (H6PDH).

Cortison reductase deficiëntie (CRD) is een zeldzame aandoening, waarbij perifere omzetting van cortison in cortisol niet plaatsvindt. Eerder was gesuggereerd dat een combinatie van twee polymorphismen, *HSD11B1* 83,557insA en *H6PD* R453Q, tot CRD zou leiden, als op zijn minst drie zeldzame allelen aanwezig waren. In **Hoofdstuk 2** laten we zien dat een aanzienlijk deel van de deelnemers van twee oudere studiepopulaties drager zijn van het 'CRD genotype' (3.8% in 'Fraild Old Men' studie en 4.0% in de Rotterdam Studie), wat niet overkomt met de extreem lage prevalentie van CRD. Bovendien vonden wij geen associaties met het 'CRD genotype' of de losse polymorphismen met glucose metabolisme, lichaamssamenstelling, bloeddruk, bijnierandrogenen en dementie.

In **Hoofdstuk 3** bestudeerden we de expressie van 11β-HSD1 in de hypothalamus en voorste hypofyse voorkwab. Beide zijn van belang in de negatieve feedback van de HPA-as. 11β-HSD1 komt in veel hypothalame kernen tot expressie, het meest prominent in de paraventriculaire kern (PVN) en supra-optische kern (SON). In de PVN komt 11β-HSD1 tot expressie in de vasopressine (AVP)-, oxytocine (OXT)- en corticotropin-releasing hormone (CRH)- producerende neuronen. Deze bevindingen suggereren dat hypothalame productie van cortisol activiteit van de HPA-as moduleert. In de voorste hypofysevoorkwab vonden we 11β-HSD1 expressie in zowel de hormoonproducerende cellen als de folliculostellate cellen. We konden niet vaststellen welke hormoonproducerende cellen 11β-HSD1 tot expressie brengen, omdat we er niet in slaagden 11β-HSD1 te visualeren met behulp van fluorescentie immunocytochemie.

In **Hoofdstuk 4** onderzochten we de effecten van veel voorkomende genetische variatie in *HSD11B1* op HPA-as activiteit met behulp van 'tagging SNPs'. Een van deze SNPs, rs11119328, was significant geassocieerd met hogere speeksel cortisolspiegels in alle deelnemers en hogere androsteendion spiegels in vrouwen. Dit past bij een verhoogde afstelling van de HPA-as.

Dragers van dit polymorphisme hadden ook een verhoogd risico op een incidente depressie. Deze bevindingen suggereren een oorzakelijk verband tussen HPA-as activiteit en depressie.

Verhoogde lokale regeneratie van cortisol door 11β-HSD1 in omentaal vet speelt mogelijk een rol in de etiologie van het metabole syndroom (MetS). In **Hoofdstuk 5** evalueerden we de effecten van veel voorkomende genetische variatie in *HSD11B1* op (kenmerken van) het MetS in twee Kaukasische studiepopulaties. In de Rotterdam Studie was rs17317033 significant geassocieerd met grotere buikomvang in vrouwen. Een zelfde trend werd gevonden in het '1966 Northern Finland Birth Cohort'. Deze studie was echter niet voldoende groot om statistisch significante verschillen aan te tonen. Geen van de andere geselecteerde SNPs was geassocieerd met MetS kenmerken of een verhoogde gevoeligheid om MetS te ontwikkelen.

Dysregulatie van de HPA-as is mogelijk een onafhankelijke risicofactor voor ischemische hartziekte. In **Hoofdstuk 6** beschrijven wij dat totale cortisol blootstelling gedurende de dag onafhankelijk geassocieerd is met aantal atherosclerotische plakken van de carotiden in een oudere populatie. De mate van cortisol afname gedurende de dag was niet geassocieerd met het aantal plakken. Prospectieve studies zijn nodig om de richting van de oorzakelijke verbanden vast te stellen.

In **Hoofdstuk 7** beschrijven we dat glucocorticoid receptor (GR) haplotypen niet bijdragen aan het risico om Guillain-Barré syndroom (GBS) te ontwikkelen. Haplotype 1, dat bestaat uit het zeldzame allel van *Bcl*I in combinatie met het wildtype allel van *TthIII*I en haplotype 2, dat bestaat uit het zowel het zeldzame allel van *Bcl*I alsook het zeldzame allel van *TthIII*I waren gerelateerd aan het klinisch beeld en prognose van GBS. Hoewel dit de grootste groep bestudeerde GBS patiënten tot nu toe was, is het toch een (te) kleine groep (n = 318) om betrouwbare genetische associaties vast te stellen. De resultaten van deze studie moeten daarom met enige voorzichtigheid geïnterpreteerd worden, totdat ze gerepliceerd worden in een andere studie.

Hoofdstuk 8 bestaat uit de algemene discussie en plaatst de resultaten beschreven in dit hoofdstuk in een breder perspectief. Specifieke aandacht wordt besteed aan de limitaties van genetische associatie studies.

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ABOUT THE AUTHOR

Marieke Dekker was born on March 8th, 1978 in Gouda. She grew up in Reeuwijk and attended secondary school at the ´Goudse Waarden´ in Gouda. Following graduation in 1996, she moved to Gent in Belgium to start her medical studies at Ghent University. She continued her medical education at the Erasmus University in Rotterdam. At the same time she followed a Master of Science Programme in Epidemiology at the Netherlands Institute of Health Sciences (NIHES) and as part of this master study she participated in the programme in Clinical Effectiveness at Harvard School of Public Health in Boston, Massachussetts. After obtaining her medical degree in November 2004 (cum laude), she started the work described in this thesis under the supervision of prof.dr. S.W.J. Lamberts, prof.dr. H Tiemeier and dr. F.J.W. Koper at the department of Internal Medicine at the Erasmus MC in Rotterdam. During this period she worked for four months at the Netherlands Institute of Neuroscience in Amsterdam to study the expression of 11β-HSD1 at the negative feedback sites of the HPA axis under guidance of prof.dr. Fliers. In 2006, she interrupted her research to take part in the ´Nationale DenkTank´ to examine the healthcare for the chronically ill in the Netherlands. In January 2009, she started as a resident internal medicine at the Harbour Hospital and Institute of Tropical diseases and in November 2010 she continued her training residencies at the Erasmus MC (supervisors dr. P.J. Wismans, Harbour Hospital, and prof. dr. J.L.C.M van Saase, Erasmus MC).

LIST OF PUBLICATIONS

Dekker MJHJ*, Smit P*, de Jong FJ, van den Beld AW, Koper JW, Pols HAP, Brinkmann AO, de Jong FH, Breteler MMB, Lamberts SWJ. Lack of Association of the 11beta-hydroxysteroid dehydrogenase type 1 gene 83,557insA and hexose-6-phosphate dehydrogenase gene R453Q polymorphisms with body composition, adrenal androgen production, blood pressure, glucose metabolism, and dementia. J Clin Endocrinol Metab 2007 92:359-62. **Both authors contributed equally.*

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Dekker MJHJ, Koper JW, Pols HAP, Hofman A, de Jong FH, Stewart PM, Tiemeier H, Lamberts SWJ. The Effect of Common Genetic Variations in 11β-Hydroxysteroid Dehydrogenase Type 1 on Hypothalamic-Pituitary-Adrenal axis activity and incident depression. *In revision.*

Bisschop PH, **Dekker MJHJ**, Osterthun W, Kwakkel J, Anink J, Boelen A, Unmehopa UA, Koper JW, Lamberts SWJ, Stewart PM, Swaab DF, Fliers E. 11β-hydroxysteroid dehydrogenase type 1 is expressed in various nuclei of the human hypothalamus and colocalizes with CRH in the paraventricular nucleus. *Submitted for publication.*

Dekker MJHJ, Bennett A, Millwood I, Koper JW, Tiemeier H, Kuningas M, Dehghan A, Groves C, Hofman A, de Jong FH, McCarthy MI, Stewart PM, Lamberts SWJ, Järvelin MR. The effect of common genetic variation in 11β-hydroxysteroid dehydrogenase type 1 gene on the metabolic syndrome. *To be submitted.*

Nationale DenkTank 2006 Recept voor morgen. *Te downloaden op www.nationale-denktank.nl.*

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- Introduction to Data-Analysis
- Regression Analysis
- Survival Analysis
- Study Design
- Introduction to Medical Writing
- Clinical Trials and Drug Risk Assessment
- Pharmaco-Epidemiology and Drug Safety
- Analysis of Time-varying Exposures
- Cancer Epidemiology

2002 Program in Clinical Effectiveness
Joint Program of Brigham and Women's Hospital, Massachusetts General Hospital, Harvard Medical School & Harvard School of Public Health, Boston, MA, USA
Courses:

- Introduction Statistics for Medical Research
- Statistics for Medical Research Advanced
- Introduction Clinical Epidemiology
- Quality Improvement in Health Care
- Society and Health

2005 Erasmus Summer Programme, Human Genetics
Courses:
- Principles of Genetic Epidemiology
- Searching Genes of Complex Disorders
- Bioinformatics in Medicine
- Genetic Epidemiology of Complex Diseases

2005 KNAW conference and Master Classes, Amsterdam, the Netherlands
´The role of DNA polymorphisms in complex traits and disease´

2007 Endocrine Society, Fellow & Students Day Workshop, Toronto, Canada

(Inter)national Conferences

2005 Dutch Endo-Neuro-Psycho Meeting 2005, Doorwerth, the Netherlands
2005 Wetenschapsdagen afdeling Interne Geneeskunde Erasmus MC (Goes)
2005 Molecular Medicine Day, Rotterdam, the Netherlands
2006 World Psychiatric Association International Congress 2006, Istanbul, Turkey
2007 Wetenschapsdagen afdeling Interne Geneeskunde Erasmus MC (Goes)
2009 Wetenschapsdagen afdeling Interne Geneeskunde Erasmus MC (Antwerpen)

Oral presentations

2007 Endocrine Society Annual Meeting 2007, Toronto, Canada
'Salivary Cortisol Secretion Is Related to Atherosclerosis of Carotid Arteries: The Rotterdam Study'
2008 International Society of Psychoneuroendocrinology, Dresden, Germany
'The Effect of Common Genetic Variation in 11β-Hydroxysteroid Dehydrogenase Type 1 Gene on hypothalamic-pituitary-adrenal axis activity'

Poster presentations

2006 Wetenschapsdagen afdeling Interne Geneeskunde Erasmus MC (Goes)
'No Association of the 11β-Hydroxysteroid Dehydrogenase Type 1 Gene 83,557insA and Hexose-6-Phosphate Dehydrogenase Gene R453Q Polymorphisms with Body Composition, Blood Pressure, Glucose Metabolism and Dementia'
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Teaching activities

2005 Mentor of 1st year medical students, Erasmus MC, Rotterdam, the Netherland

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