Variation in the Glucocorticoid Receptor Gene: Consequences for Body and Brain

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Variation in the Glucocorticoid Receptor Gene: Consequences for Body and Brain

Variatie in het Glucocorticoid Receptor Gen:
Gevolgen voor Lichaam en Brein

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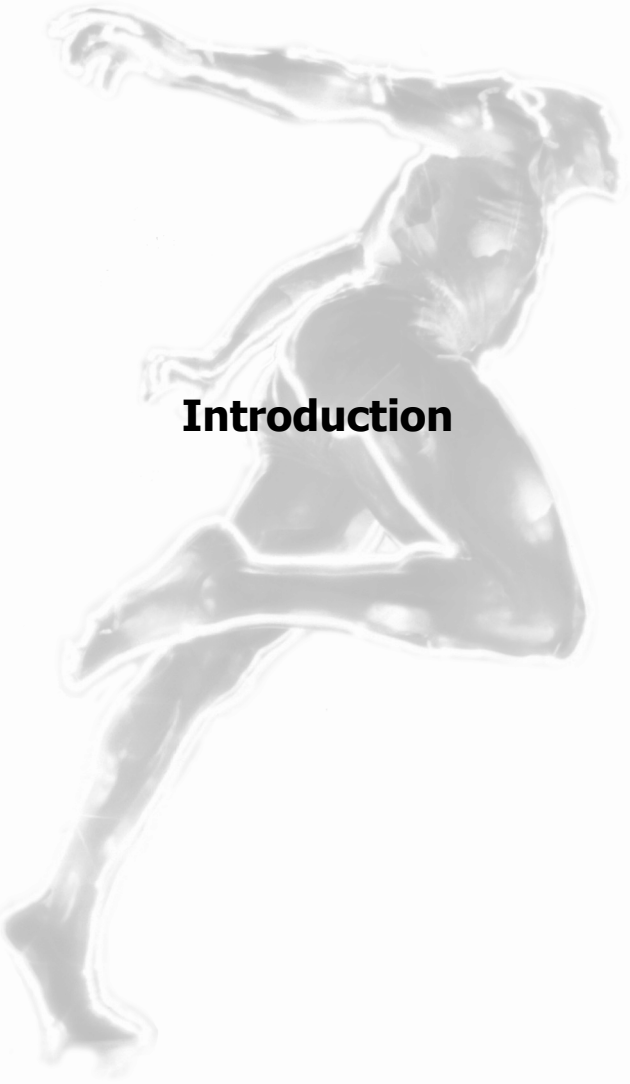

Introduction

Introduction

1.1 Regulation of the Hypothalamic-Pituitary-Adrenal-axis

The secretion of glucocorticoids (GCs) is regulated by the hypothalamus, which receives stimuli from the central nervous system [1]. In response to these stimuli (e.g. physical or psychological stress) neurons in the paraventricular nucleus of the hypothalamus secrete corticothopin releasing hormone (CRH) and its cosecretagogue vasopressin [2]. As a result the pituitary is stimulated to secrete corticotropin (ACTH) [3], which is synthesized as part of a large precursor, pro-opiomelanocortin (POMC) [4]. ACTH stimulates the adrenal glands to produce glucocorticoids. The major glucocorticoid in humans is cortisol. This cascade of the hypothalamus-pituitary-adrenal (HPA) axis results in a diurnal profile of cortisol secretion with high levels in the morning and low concentrations in the afternoon and evening, with a small peak after lunch.

Important in the regulation of the production of GCs is the negative feeedback action by GCs. GCs inhibit hormone synthesis and secretion both at the level of the hypothalamus and the pituitary. In the corticotropic cell, GCs have inhibitory effects on both POMC gene transcription and ACTH secretion [4]. Also, GCs decrease CRH and AVP mRNA levels in the hypothalamic paraventricular nuclei [5-7]. A third mechanism by which GCs exert a negative effect on their own production is blockade of the stimulatory effect of CRH on POMC gene transcription [4]. Figure 1 shows a simplified model of the HPA axis.

1.2 The effects of Glucocorticoids

Cortisol has numerous effects throughout the human body, including the mediation of the stress response, regulation of lipid and glucose metabolism, immunosuppressive and anti-inflammatory actions, vascular effects, increase of bone resorption, as well as effects on the development and function of numerous organs. Because of the suppressive effects on the immune system GCs are widely used in the treatment of diseases in which inflammation (e.g. inflammatory bowel disease) or the immune system (e.g. asthma, rheumatoid arthritis) play an important role, as well as in the prevention of rejection of organ transplants. When present in excess, due to endogenous overproduction (e.g. Cushing's disease) or therapeutically administrated GCs, serious adverse effects can occur, as listed in table 1. Increased levels of cortisol, as a result of HPA axis overactivity, which is related to stress [8], has been associated with cognitive impairment and dementia [9, 10]. Longitudinal studies in both Alzheimer's disease (AD) patients and healthy elderly showed higher plasma cortisol levels leading to a more rapid decline in cognitive function over time [10-12]. Increasing age has been shown to be associated with elevated evening cortisol levels in men [13, 14]. An increased exposure of several tissues to glucocorticoids with aging, e.g., visceral fat cells, in combination with the reduction of the lipolytic effects of declining growth hormone levels, may contribute to the age-dependent

increase of visceral fat accumulation. In addition, HPA axis overactivity is related to an increased vascular risk, including hypertension and obesity [15, 16]. A well-known effect of glucocorticoids is to negatively influence body composition, including redistribution of body fat with deposition of adipose tissue on the abdomen and trunk, and muscle atrophy [17]. Longterm exposure to high levels of glucocorticoids induces loss of muscle mass and inhibits growth [18, 19]. It is known that body composition plays an important role in lipid metabolism and insulin sensitivity, and as a consequence influences the risk on cardiovascular disease [20].

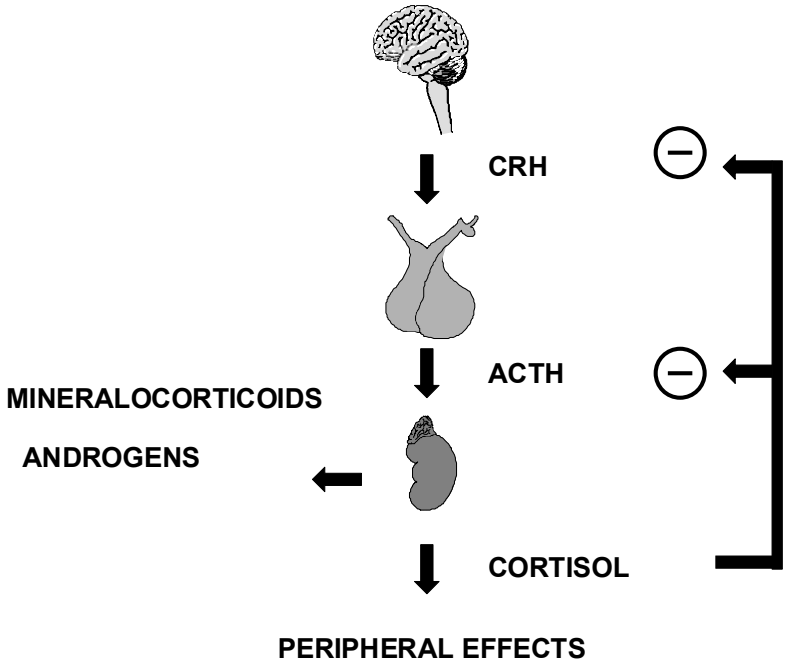

Figure 1: A simplified overview of the regulation of GCs by the HPA-axis. Under the influence of stress (both physiological and psychological) and the circadian rhythm the hypothalamus secretes CRH and AVP into the hypophysial portal system and thereby stimulates the production of ACTH by the pituitary. In response to increased levels of ACTH the adrenal glands increase the secretion cortisol along with other adrenal steroids with mineralocorticoid and androgen activity. Cortisol inhibits its own production both at the hypothalamic and pituitary level and thereby completing a negative feedback loop.

1.3 Sensitivity to Glucocorticoids

It is known, that the sensitivity to exogenous GCs is highly variable between patients. Some patients have excellent responses with respect to their diseases, but also suffer from severe adverse effects, while others need a very high dose to exert any therapeutical effect at all. When examined with a dexamethasone (DEX) suppression test a similar variability in the sensitivity to GCs between different normal individuals [21] is observed as well. However, within individuals GC sensitivity is rather stable. This suggests that, in humans, a setpoint for the sensitivity to DEX with respect to the feedback action exists, which might be genetically determined. An important factor in the cascade of GC action, also at the pituitary level, is binding to the GC receptor (GR). The GR belongs to the superfamily of nuclear receptors, which are present in the cytoplasm and act as transcription factors to regulate gene expression. After binding of cortisol, a conformational change occurs, which leads to dissociation of the receptor from a large complex of proteins of which heat shock protein 90 is the most important [22, 23]. This activated ligand-bound receptor then translocates to the nucleus where it can act in several ways [24], as shown in figure 2. The GR can initiate transcription through binding to GC response elements of the target gene. The GR also can affect gene transcription through direct protein-protein interaction and can activate, as well as repress target gene expression [25 26 27]. In mice, in which a mutation was induced which impaired dimerization and DNA-binding, it has been shown that these processes are not critical for survival [28]. Previously, some rare mutations of the GR gene have been described (see Figure 3), which led to clinical signs and symptoms of generalized cortisol resistance [30]. Due to these receptor defects, cortisol has impaired actions through the GR. As a consequence, the central negative feedback of GCs is diminished, GC production by the adrenal is increased. Cortisol binds with high affinity to the mineralocorticoid receptor [31]. Symptomatology in patients with cortisol resistance are the consequence of a compensatory hyperactivity of the HPA-axis, which results in an overproduction of mineralocorticoids, which in turn lead to: hypertension, hypokalemic alkalosis, fatigue, and in females due to higher adrenal production of androgens, also hyperandrogenism. In normal conditions, organs which have an important mineralocorticoid function are protected from high cortisol levels by the enzyme 11β-hydroxysteroid dehydrogenase type II (11β-HSD II), which rapidly inactivates cortisol in to cortisone. In the case of cortisol resistance, cortisol levels are too high for the inactivational capacity of this enzyme. The number of patients diagnosed with cortisol resistance syndrome until now is low (in about 10 patients) [32-39]. Also, two mutations were found in vitro, which could have been preexisting acquired mutations in vivo, which lead to Nelson syndrome and lupus nephritis [40, 41]. Most patients carried a mutation or defect in the ligand-binding domain and just one patient had a mutation in the DNA-binding domain [42]. A possible explanation for the low number of patients is that a severe form of cortisol resistance is not compatible with life.

Table 1: Major side effects associated with glucocorticoid therapy

Major side effects associated with Glucocorticoid Therapy	
Dermatologic and soft tissue	**Renal**
Skin thinning and purpura	Hypokalemia
Cushingoid appearance	Fluid volume shifts
Alopecia	**Genitourinary and reproductive**
Acne	Amenorrhea/ infertility
Hirsutism	Intrauterine growth retardation
Striae	**Bone**
Hypertrichosis	Osteoporosis
Eye	Avascular necrosis
Posterior subcapsular cataract	**Muscle**
Elevated intraocular pressure/glaucoma	Myopathy
Exophthalmos	**Neuropsychiatric**
Cardiovascular	Euphoria
Hypertension	Dysphoria/ depression
Perturbations of serum lipoproteins	Insomnia
Premature atherosclerotic disease	Psychosis
Gastrointestinal	**Endocrine**
Gastritis	Diabetes mellitus
Peptic ulcer disease	HPA insufficiency
Pancreatitis	**Infectious disease**
Steatosis hepatis	Increased risk of typical infections
Visceral perforation	Opportunistic infections

HPA, hypothalamic-pituitary-adrenal

MODES OF ACTION OF THE GLUCOCORTICOID RECEPTOR

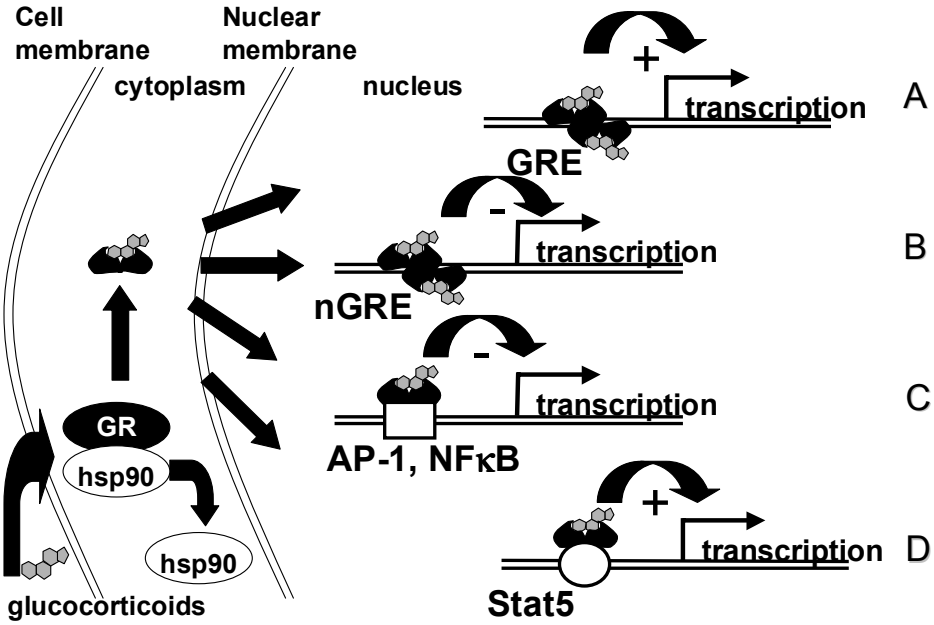

Figure 2 *(adapted from Reichardt and Schütz [29]): The first step leading to induction of gene expression by GCs is binding of the hormone to the receptor. Since cortisol is lipophilic it is thought to enter the cell by passive diffusion. After binding the hsp-complex dissociates, hsp 90 is released and the domains responsible for nuclear localisation, DNA binding and transactivation are unmasked. The receptor becomes hyperphosphorylated and forms homodimers (as result of the interaction of dimerization sequences present in the ligand and DNA binding domains.) GR molecules translocate to the nucleus. Inside the nucleus the activated GR-dimers can act in several ways.*

A. *the first way represents the classical model for GR action: the receptor homodimer binds to short, palindromically arranged DNA sequences in the promotor region of the GC responsive genes, which are called GC responsive elements (GREs). The receptor homodimers bind to DNA using their zinc fingers. These structures of the DNA binding domains form a finger-like loop structure of 12 amino-acids, which interact with the coils of the DNA double helix. When bound to the GRE, the receptor homodimer can interact with the basic transcription cascade in several ways. One possibility is a direct interaction via contact between the GR transactivation domains and transcription factors. Furthermore, binding of the GR homodimer to the GRE can induce a chromatin structure*

rearrangement allowing other transcription factors to bind to the previously inaccessable DNA.

B. *In some promoters, POMC is a prototype example, binding of the activated GR to the GRE induces transcription inhibition, rather than activation. These GREs therefore are called negative GREs (nGRE).*

C. *Another way to function is through direct protein-protein interaction. These genes are positively regulated by activating protein 1 (AP-1), which is a transcription factor that consists of dimers of the Fos and Jun protein family. GR probably interacts directly with AP-1 and prevents its activating action.*
A similar pattern has been reported for the transcription factor nuclear factor κ B (NF-κB). The GR mediated transrepression of NF-κ B is caused by interaction with one of the subunits of NF- κB.

D. *Another direct protein protein action is exerted by interaction of GR with Stat 5 which positively regulates transcription*

Hypersensitivity to endogenous cortisol has been described as well: Iida et al reported a patient with symptoms of Cushing's syndrome, despite hypocortisolemia [43]. More recently, Newfield et al described a second patient with serious symptoms of Cushing's syndrome at peripubertal age, but normal cortisol levels [44]. The lymphocytes of this second patient contained an increased number of GR per cell with normal binding affinity. The molecular etiology of hyperreactivity to cortisol has not been fully clarified yet. Figure 3 shows a schematic overview of the GR gene and the locations of the previously described mutations causing cortisol resistance, and polymorphisms which have been shown to be associated with an altered sensitivity to GCs. In contrast to the infrequent mutations, the majority of polymorphisms are located in the N-terminal transactivation domain [45]. This thesis deals with GR gene polymorphisms, which were not only associated with differences in GC sensitivity, but as a result also were related to differences in body composition and metabolic parameters.

1.4 Polymorphisms of the Glucocorticoid Receptor Gene

The N363S Polymorphism of the GR gene

Previously, a polymorphism was identified in codon 363 of exon 2 of the GR gene (Figure 3). Table 2 shows an overview of the associations with body mass index and metabolic parameters found with this polymorphism so far. This AAT to AGT nucleotide change results in an asparagine to serine amino acid change and appeared in a group of 216 normal Dutch elderly individuals to be associated with a higher sensitivity to GCs in vivo [46, 47]. This was shown by

lower cortisol levels after the administration of 0.25 mg DEX, as well as a significantly greater decrease in cortisol levels. Moreover, in this population N363S-carriers also had an increased insulin response to exogenous DEX, which is likely to be directly related to their increased GC sensitivity. In addition, N363S-carriers had a higher body mass index (BMI), and a tendency towards decreased bone mineral density in trabecular bone [47, 48]. Lin et al confirmed the association with BMI, and even demonstrated an allele-dosage effect on BMI (homozygous S-allele carriers had a higher BMI than heterozygous S-allele carriers) [48].

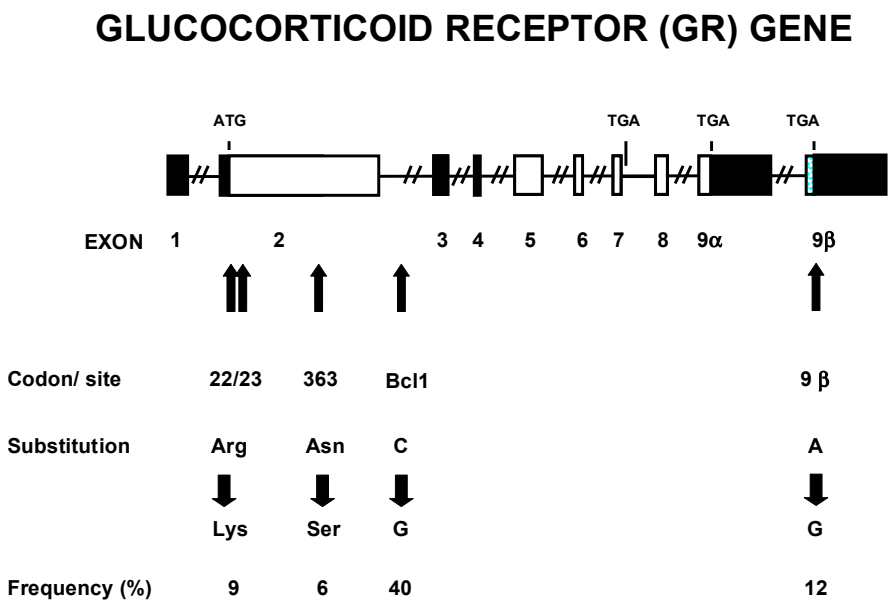

Figure 3: *Schematic overview of the GR gene, showing polymorphisms (black arrows) and their locations, nucleotide and/or amino acid substitutions and frequencies.*

However, thereafter some controversy arose concerning the role of this polymorphism, as recently reviewed by Rosmond [49]. Dobson et al found an increased waist-to-hip ratio in male N363S-carriers, but no associations with BMI, serum lipid levels and glucose tolerance status in a Caucasian population [50]. In three other reports no association was observed between the N363S polymorphism and BMI either [51-53]. In a recent report of Lin et al the N363S variant was associated with coronary artery disease independent of weight [54]. The frequency of the 363S-allele was in particular high in patients with angina pectoris. In this population of Anglo-Celtic descent, also several risk factors of atherosclerosis were associated with the N363S variant: increased cholesterol and triglyceride concentrations, as well as an increased total

cholesterol/HDL-cholesterol ratio. Recently, the same authors showed again an association between the N363S polymorphism and obesity, as well as overweight in several groups of patients [55].

Table 2: Data from 8 studies that investigated the association between the N363S polymorphism of the *GR* gene and body mass index

Reference	Population	Associations with the N363S polymorphism
Huizenga et al, 1998 [47]	216 Dutch men and women	Increased GC sensitivity , increased insulin response to DEX, increased BMI
Lin et al, 1999 [48]	195 normotensive controls and 124 hypertensive subjects	Increased BMI, allele-dosage effect
Halsall et al, 2000 [53]	491 subjects	No association with BMI
Dobson et al, 2001 [50]	135 men and 240 women	Increased WHR in men
Rosmond etal, 2001 [52]	284 Swedish men	No association with BMI No association with sensitivity to GCs
Echwald et al, 2001 [51]	741 obese Danish men and 854 non-obese controls	No association with BMI, WHR or weight gain
Lin et al, 2003 [54]	437 Anglo-Celtic CAD patients and 302 controls	Association with CAD, elevated cholesterol, triglycerides, total cholesterol/HDL ratio
Lin et al, 2003 [55]	951 Anglo-Celtic/Northern Europe subjects: 152 obese, 356 type 2 diabetes, 141 hypertensive, 302 controls	Association with obesity and overweight in several patient settings, but no association with hypertension or type 2 diabetes

BMI, body mass index, CAD, coronary artery disease, DEX, dexamethasone, GC, glucocorticoid, HDL, high density lipoprotein -cholesterol , WHR, waist-to-hip ratio

However, no association was found with hypertension or type 2 diabetes. Interestingly, in a Japanese, as well as in a Chinese population the N363S variant did not occur [56, 57]. In this thesis we studied whether the N363S variant is associated with BMI and cholesterol levels in an Italian severely obese population. In addition, we investigated the combined effects of carrying both the N363S and the BclI polymorphisms in these obese patients.

The BclI Polymorphism of the GR gene

Murray et al reported an intronic RFLP of the *GR* gene, which was described as consisting of a short fragment of 2.3 kb and a large fragment of 4.5 kb [58]. Since then, several association studies were performed to investigate the role of this variant in obesity using the terminology and technique Murray used. We investigated the exact nucleotide alteration. Table 3 shows an overview of the reports sofar of the *Bcl*I polymorphism and its associations with body composition and metabolic parameters. The first association study of the *Bcl*I polymorphism which was decribed by Weaver et al, showed no differences in frequency of the *Bcl*I polymorphism between an obese and a normal-weight population [59]. However, within the obese group, homozygous G-allele (4.5 kb) carriers had higher insulin levels and were more insulin resistant when compared to a group consisting of CC (homozygous 2.3 kb) and CG (2.3/4.5 kb)-carriers. In a report of Panarelli et al no association between the G-allele and BMI was described either [62]. However, increased skin vasoconstriction was observed in homozygous G-allele carriers after injection with budesonide, a synthetic GC, which suggests increased i*n vivo* sensitivity to GCs. In contrast, in this study it was shown the *in vitro* affinity and sensitivity of leucocytes to dexamethasone tended to be lower. Although these findings were not statistically significant, it suggests that this polymorphism might have tissue-specific effects. Three other reports, all in middle-aged individuals, showed an association of the *Bcl*I polymorphism with abdominal visceral obesity, but not with general obesity [63, 64, 66] GCs are known to induce central obesity, as is observed in Cushing's disease. Sofar, it is not known whether this polymorphism is also associated with other features of Cushing's syndrome e.g. easy bruisability. However, the relationship between abdominal obesity and the *Bcl*I polymorphism suggests a greater effect of GCs due to alterations at the level of the GR, in particular in visceral fat.

In an experiment of 100 days which was conducted with 12 pairs of monozygotic twins at young adult age, the effects of the *Bcl*I variant were studied in relation to body composition and metabolic changes in response to overfeeding [65]. In this study no homozygous G-allele carriers were found. In contrast with the findings of the above discussed reports, CC-carriers experienced a greater increase in body weight, visceral fat and cholesterol levels after overfeeding than CG-carriers. However, another study in adolescents showed in female heterozygous CG-allele carriers a greater increase in subcutaneous fat, as measured by

TABLE 3. Data from studies that involved the BclI Polymorphism of the GR Gene and that investigated whether there were differences between CC-carriers (in previous reports described as homozygous 2.3 kb-allele carriers), CG-carriers (heterozygous 2.3/4.5 kb carriers), and GG-carriers (in previous reports: homozygous 4.5 kb-allele carriers) in body composition, blood pressure and metabolic parameters.

Reference	Population	Associations with the G-allele of the *Bcl*I polymorphism
Weaver et al, 1992 [59]	56 obese and 43 nonobese premenopausal women	Hyperinsulinemia in obese GG-carriers, but not in non-obese GG-carriers, no association of the G-allele with obesity
Watt et al, 1992 [60]	864 adults (aged 16-24 yrs) and their parents	Homozygosity for the G-allele was more frequent in the group with personal and parental hypertension
Clement et al, 1996 [61]	80 obese families	Tendency towards linkage between the *Bcl*I marker and obesity (BMI>27), no association after replication
Panarelli et al, 1998 [62]	64 men (aged 18-40 yrs)	No association of the G-allele with BMI, Increased in vivo sensitivity to budesonide in GG-carriers
Buemann et al, 1997 [63]	79 men and 73 women, middle-aged	Increased abdominal visceral fat in lean GG-carriers, but not in overweight GG-carriers.
Rosmond et al, 2000 [64]	262 Swedish men	Increased abdominal obesity and higher cortisol levels in GG-carriers compared to CC-carriers
Ukkola et al, 2001 [65]	12 pairs of monozygotic lean male twins (aged 21 yrs)	CC-carriers had a greater increase in weight, abdominal visceral fat and cholesterol levels in response to overfeeding compared to CG-carriers
Ukkola et al, 2001 [66]	322 men and 420 women (aged 42 yrs)	G-allele associated with abdominal visceral fat and gene-gene interactions present with lipoprotein lipase gene and adrenergic receptor gene
Tremblay et al, 2003 [67]	90 male and 83 female adolescents	Female CG-carriers had a greater increase in subcutaneous fat mass during a 12-yr follow-up than CC- carriers or GG-carriers. No differences were found in males.

skinfolds, when compared to both homozygous CC- and GG-carriers during a 12 years follow-up period [67]. No differences were found in baseline or post follow-up subcutaneous fat mass, or in total fat mass or, importantly, trunk fat mass. The authors speculated that one mutated allele could have a different effect than two mutated alleles. In the latter state an alternative pathway might be switched on to compensate for changes resulting from two polymorphic alleles. As these authors refer to, mechanisms supporting this theory, have indeed been reported in mice models involving the cyclooxygenase-2 and the glucose transporter-4 genes [68] [69]. It is known that body composition changes during the normal aging process, including increased sarcopenia. In this thesis we identified the exact sequence alteration of the BclI polymorphism and describe the effects of this variant on GC sensitivity as well as the effects on body composition and bone mineral density at older age.

The ER22/23EK Polymorphism of the GR gene

In a previous report a polymorphism, consisting of 2 linked single nucleotide mutations in codons 22 and 23 (exon 2 of the GR gene, figure 3) was described [46]. The first mutation in codon 22 did not result in an amino acid change (GAG to GAA, both coding for a glutamic acid (E)), but the mutation in codon 23 (AGG to AAG) causes a change from arginine (R) to lysine (K). We investigated the effects of this polymorphism on GC sensitivity using a dexamethasone suppression test of 0.25 mg and of 1 mg, as well as the effects on insulin and lipid status. We extended these studies to investigate a possible relation to predictors of mortality (C-reactive protein and interleukin-6) and studied survival in a population of elderly men. To investigate whether this GR variant is also associated with changes during puberty, we studied the effects on well-defined measures as anthropometric parameters, body composition and muscle strength at young age.

It is known that GCs influence important brain structures and a correct level of cortisol is critical for many cerebral functions. In humans it has been shown that high cortisol levels resulted in a decreased hippocampal formation volume, and memory impairment [70] [71]. Also, disturbances in the hypothalamic-pituitary-adrenal axis have been found to be related to dementia disorders [72] [73] [74] In a large population-based study in the elderly we studied whether the ER22/23EK polymorphism was associated with hippocampal volume, dementia and white matter lesions.

The TthIIIΙ Polymorphism of the GR gene

In the promoter region of the *GR* gene a *TthIIIΙ* RFLP was previously reported by Detera-Wadleigh et al [75] (Figure 3). Rosmond et al showed an association of this polymorphism with elevated diurnal cortisol levels in a population of 284 Swedish men [76]. No relationships were

found between the *TthIII*I variant and anthropometry, glucose and insulin metabolism or lipid spectrum. In this thesis we characterized the exact location of the nucleotide change. In the same subpopulation of the Rotterdam Study in which we studied the relationship between the three other polymorphisms described in this thesis and feedback sensitivity to GCs, we investigated whether there was an association between the *TthIII*I polymorphism and GC sensitivity, insulin and lipid metabolism and anthropometric parameters.

The exon 9 beta Polymorphism of the GR gene

The GR gene consists of 10 exons. Exons 1-9α are transcribed to GRα mRNA, which is translated to the functional GRα. To a small extent alternative splicing of the primary transcript occurs, which results in an mRNA consisting of exons 1 to 9β, which is translated to GRβ [77, 78]. This alternate protein GRβ does not bind ligand and is not transcriptionally active. It has been shown that in vitro this GRβ can function as a dominant negative inhibitor of the active GRα [79, 80]. However, these findings remain controversial, since several other studies could not reproduce these results [81-83].

Previously, DeRijk et al reported an A to G substitution in an "ATTTA motif" in exon 9β of the GR gene [84]. This variable ATTTA (to GTTTA) sequence is located in a region encoding the 3' untranslated region (UTR) of the GRβ mRNA. A stabilizing effect of this polymorphism on the GRβ mRNA was observed.[84]. In the same study an association between this exon 9β polymorphism and rheumatoid arthritis was found. As suggested by DeRijk et al this polymorphism could result in an increased expression and stability of GRβ in vivo, and consequently lead to glucocorticoid resistance in rheumatoid arthritis patients.

1.5 The Hypothalamo-Pituitary-Adrenal Axis and Depression

Another topic we address in this thesis is depression. Hyperactivity of the hypothalamic-pituitary adrenal (HPA)-axis seems to be important in the pathogenesis of depression. The normalization of the HPA-axis is a necessary predecessor of clinical response to antidepressant therapy [85]. An impaired signaling pathway via glucocorticoid receptors (GR), leading to an impaired negative feedback regulation and thus to partial glucocorticoid resistance appears to cause this hyperactivity. In depressed patients this is reflected by a basal hypercortisolemia and cortisol escape from dexamethasone suppression [86], as well as an increased ACTH and cortisol release in the combined dexamethasone suppression/CRH-stimulation test (Dex-CRH test) [87-89].

On the other hand, increased GR activation may also promote depressive symptoms. In Cushing's disease, characterized by severely increased cortisol levels, symptoms of depression frequently occur [90]. In addition, glucocorticoids exert a positive feedback on CRH expression in limbic regions such the amygdala [91, 92]. Increased CRH neurotransmission in limbic regions has been associated with increased depression-like symptomatology [92].

Therefore, not only glucocorticoid resistance seems to be related to depression, but also enhanced GR effects in limbic brain regions may contribute to the development of depression.

There are data suggesting that HPA-axis hyperactivity is a heritable trait, since mentally healthy first-degree relatives of depressive patients also show an increased reactivity of the HPA-axis in response to the Dex-CRH test [93, 94]. Genetic factors leading to an altered GR sensitivity may therefore alter the susceptibility to depression and the response to antidepressant drugs. In addition, functional variants in the GR gene are also likely to affect the outcome of neuroendocrine tests, such as the Dex-CRH test, and neuropsychological tests in depressed patients. In this thesis we investigated the role of GR polymorphisms in depression.

1.6 Aims of this thesis

A considerable variability in the response to both exogenous as well as to endogenous GCs exists between normal individuals. We investigated the role of polymorphisms of the GR gene (ER22/23EK, N363S, BclI, TthIII) in this variability in sensitivity to GCs using a dexamethasone suppression test of 1 mg and of 0.25 mg. In addition, we studied the effects of these genetic GR variants on metabolism: lipid levels, insulin sensitivity and markers of inflammation (interleukin-6 and C-reactive protein), which seem to be involved in the process of atherosclerosis [95]. It is known that body composition plays an important role in lipid metabolism and insulin sensitivity, and as a consequence influences the risk on cardiovascular disease [20]. A well-known effect of glucocorticoids is to negatively influence body composition, including redistribution of body fat with deposition of adipose tissue on the abdomen and trunk, and muscle atrophy [17]. In this thesis we also studied the effects of the GR polymorphisms on body composition, height and muscle strength.

HPA axis overactivity, which is related to stress leads to increased levels of cortisol [8], and has been associated with cognitive impairment and dementia [9, 10]. In this thesis we studied whether the ER22/23EK polymorphism was associated with hippocampal volume, cognive impairment and dementia, as well as cerebral white matter lesions. Hyperactivity of the HPA-axis seems also to be important in the pathogenesis of depression. A predecessor of clinical response to antidepressant therapy is the normalization of the HPA-axis [85]. An impaired negative feedback regulation and thus a partial glucocorticoid resistance appears to cause this hyperactivity, which might be partially genetically determined [93, 94]. Genetic polymorphisms leading to a alterered GR sensitivity may therefore alter the susceptibility to depression and the response to antidepressant drugs. In this thesis we investigated whether GR polymorphisms are associated with major depression, as well as outcomes of neuroendocrine tests, such as the Dex-CRH test, and neuropsychological tests in depressed patients, and the response to antidepressant treatment. In the general discussion limitations of polymorphism studies, as well

as the associations between GR polymorphisms and these measures of GC sensitivity, body composition, metabolism, cognition, brain structures and depression are discussed.

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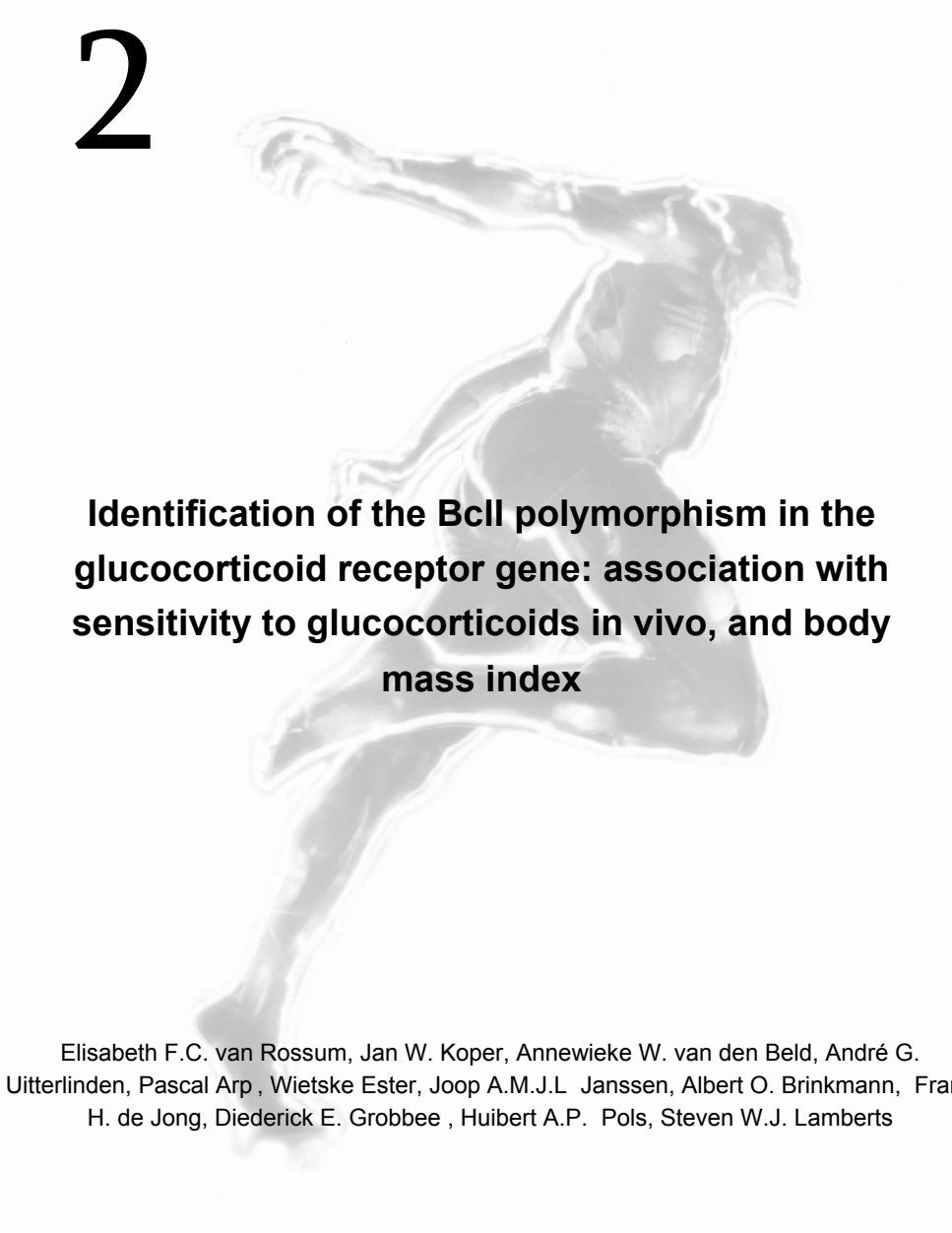

Identification of the BclI polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo, and body mass index

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Abstract

Objective: Sensitivity to glucocorticoids differs between individuals, partially due to genetic variation in the glucocorticoid receptor (GR) gene. We studied the sequence alteration of a previously described intronic *BclI* polymorphism of the *GR* gene, and investigated whether there was an association with sensitivity to glucocorticoids and anthropometric parameters in a group of healthy elderly individuals. Design and measurements: in study group 1, two overnight dexamethasone suppression tests (DSTs) were performed: with 1 mg dexamethasone, and 2.5 years later with 0.25 mg dexamethasone. Anthropometric parameters were measured in a larger population (study group 2) , as well as in a third study group, in which we also measured body composition by DEXA scans. Subjects: group 1 and 2: respectively 191 and 1963 male and female participants of the Rotterdam study, a population-based study in Dutch elderly. Study group 3: 370 elderly males (mean age 77.8 ± 0.2 yrs) from Zoetermeer, The Netherlands. Results: we identified the *BclI* restriction site polymorphism as a C/G substitution in intron 2, 646 nucleotides downstream from exon 2. After both 1 mg and 0.25 mg DST, heterozygous (CG) and homozygous G-allele carriers (GG) had lower cortisol levels than CC-carriers (p=0.01, p=0.02, respectively). In study group 2 we found a lower BMI (p=0.006) and WHR (p=0.02) in G-allele carriers. In study group 3, again we found a lower BMI (p=0.05) in G-allele carriers. No differences were found in fat mass. However, lean mass tended to be lower in G-allele carriers (p=0.07). Conclusions: we characterized a *BclI*-RFLP of the *GR* gene as a C/G polymorphism in intron 2 of which the G-allele was associated with hypersensitivity to glucocorticoids. This resulted in a lower BMI in older individuals in general, while our study in elderly males suggests that the lower BMI is probably due to a greater loss of lean mass during the ageing process.

Introduction

Sensitivity to endogenous or exogenous glucocorticoids (GCs), hormones with important regulatory effects throughout the human body, is known to show a large interindividual variation [1]. The effects of the GCs are mediated via the glucocorticoid receptor (GR). Clinically, some patients appear more sensitive to the therapeutic administration of GCs than others. The molecular etiology of cortisol hyperreactivity has not been fully clarified yet, but a single nucleotide polymorphism of the *GR* gene in codon 363, resulting in an asparagine to serine amino acid change is associated with a higher sensitivity to GCs in vivo [2, 3]. The S-allele of the N363S polymorphism has been associated with a higher body mass index (BMI), increased cardiovascular risk, increased insulin response to exogenous dexamethasone (DEX) and a tendency towards decreased bone mineral density in trabecular bone [3, 4]. However, the role of this polymorphism is still controversial, as recently reviewed [5].

Another variant of the *GR* gene, a *BclI* restriction fragment length polymorphism (RFLP) presumably located in intron 2 [6, 7] was described by Murray et al. The 4.5-kb fragment was found to be associated with abdominal obesity, higher systolic blood pressure, as well as elevated cortisol concentrations after a standardized lunch, but no association with respect to the response to a 0.5 mg dexamthasone suppression test. [6, 8, 9]. In a group of young adult males, this polymorphism was associated with an increased reaction to a skin vasocontriction test with budesonide, but not with differences in blood pressure or in *in vitro* experiments concerning the affinity and concentration of GR in leucocytes [10]. Furthermore, Weaver et al reported that premenopausal obese women that were homozygous for the 4.5 kb allele had increased fasting insulin levels and a higher insulin-resistance index [11]. The frequency.of the 4.5 kb allele, however, was not different between these obese women and a normal-weight control group. Another study in 12 pairs of identical twins showed that homozygous carriers of the 2.3 kb allele increased more in body weight, abdominal fat mass and cholesterol levels after a period of overfeeding when compared to heterozygous 2.3/4.5 kb allele carriers [12]. All these studies were performed in young or middle-aged populations. It is unknown whether there is an effect of the *BclI* polymorphism at older age. In addition, at present it remains unclear what the effects of this polymorphism on sensitivity to GCs are. Furthermore, most results from other studies are from relatively small sample-sizes, probably because the exact mutation was not known and the method of Southern blotting is rather labour-intensive.

In the present study we identified the sequence alteration detected as the *BclI* RFLP and we present evidence that the G-allele of this *BclI* polymorphism is associated with hypersensitivity to GCs and differences in body composition.

Methods and subjects

Subjects

Study group 1: for the cortisol and insulin measurements, a total of 191 subjects were randomly selected from the Rotterdam Study, a population-based cohort study (7983 subjects) in a suburb of Rotterdam, The Netherlands, in whom the determinants of chronic disabling diseases in the elderly are studied [3]. Subjects with acute, psychiatric or endocrine diseases, including diabetes mellitus treated with medication, were not invited. Three subjects were taking estrogen-containing medication and were excluded from the analysis because of the significant effect on corticosteroid globulin and therefore on cortisol. In one male subject no dexamethasone (DEX) was measurable, suggesting that he had not taken the 1 mg DEX tablet, therefore he was excluded as well. Age in this study group varied between 53 and 82 (91 men and 100 women with mean ages of 67.7 ± 0.6 and 65.9 ± 0.6 years, respectively). In order to get more information about the individual variability of the feedback sensitivity of the hypothalamo-pituitary-adrenal (HPA)- axis, the 191 subjects, who underwent a 1 mg dexamethasone suppression test (DST), were invited again two and a half years later for a second DST with a lower dose of DEX (0.25 mg). 143 (74.1%) subjects agreed to participate in this second test (67 men and 76 women).

Study group 2: Anthropometric parameters and bone mineral density were studied in a group of 1963 participants of the Rotterdam study. For this study group we included independently living subjects, who were excluded according to the following criteria: use of a walking aid, known diabetes mellitus type II, age over 80 years and use of thyroid hormone, chemotherapeutic drugs, or diuretics. Their age varied between 55 and 80 years (933 men and 1030 women with mean ages of 67.3 ± 0.2 and 67.2 ± 0.2 years, respectively).

Study group 3: Body composition was studied in more detail in a group of 370 indepently living men, aged 73 yr or older. Participants were recruited by a letter of invitation, which were sent to the oldest male inhabitants of Zoetermeer, a medium-sized town in the Netherlands. Subjects were judged sufficiently healthy to participate in the study if they were physically and mentally able to visit the study center independently. No additional health-related eligibility criteria were used. All subjects gave their written informed consent to participate in the study which received the approval of the Medical Ethics Committee of the Erasmus MC.

Anthropometric Measurements Body weight, height and waist to hip ratio of the subjects were measured, and the body mass index (BMI, kg/m^2) was calculated. Blood pressure was measured in sitting position at the right upper arm with a random-zero sphygmomanometer.

Body composition Measurements Total fat mass, trunk fat mass and lean body mass were measured in study group 3 using dual energy x-ray absorptiometry (DEXA, Lunar Corp., Madison, WI) [13]. Quality assurance for DEXA, including calibration, was performed routinely every morning, using the standard provided by the manufacturer.

Bone mineral density Bone mineral density (BMD) measurements were performed by DEXA, using a DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA). Standard positioning was used with anterior- posterior scans of the lumbar spine and the right proximal femur. In cases of a history of hip fracture or prothesis implantation, the left femur was scanned. Using standard software the vertebrae L2 to L4 and at the proximal femur, the femoral neck, and the greater trochanter were analysed. Quality assurance included calibration with the standard of the machine, and was performed routinely every morning. The in vivo coefficient of variation for the BMD measurements was 0.9% in the lumbar spine, 3.2 % in the femoral neck, and 2.5 % in the greater trochanter [14].

Dexamethasone suppression tests The two dexamethasone suppression tests (DST) were performed as described previously [15]. In brief, venous blood was obtained between 8 and 9 am after an overnight fast for serum cortisol and insulin measurements. Participants were instructed to ingest a tablet of 1 mg (or 0.25 mg for the second DST) DEX at 11.00 pm. The next morning fasting blood was drawn by venapuncture at the same time as the previous morning. To check for compliance and possible abnormalities in the metabolism of DEX, the DEX concentration was also measured by a radioimmunoassay.

Hormonal Measurements Serum cortisol concentrations were determined using RIA-kits obtained from Diagnostics Products Corporation (Los Angeles, CA). Intra- and interassay variations were below 8.0 % and 9.5 % respectively. Circulating insulin concentrations were measured using commercially available radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium). Intra- and interassay variations were 8.0 % and 13.7 % respectively.

Sequencing analysis A *Bcl*I recognition site possibly involved in the *Bcl*I RFLP [7] was identified in Genbank sequence NT_030707. A fragment including this site was PCR amplified from 10 random DNA samples using the primers 5'-GCTCACAGGGTTCTTGCCATA-3' (forward) and 5'-TTGCACCATGTTGACACCAAT-3' (reverse). The PCR fragments were digested with BclI enzyme (New England Biolabs Ltd, UK) and analyzed on agarose gels. The *Bcl*I site was indeed found to be polymorphic. Subsequently, the sequence of the fragments was analyzed: purified PCR products were sequenced on a ABI Prism 310 Genetic Analyzer, using a BigDye™ Terminator Cycle Sequencing Ready Reaction DNA sequencing kit (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands) according to manufacturer's protocol.

Genetic analysis DNA of the 191 persons in the first study group and of the 370 subjects of the third study group was extracted from samples of peripheral venous blood according to standard procedures. Genotyping was performed by allelic discrimination using TaqMan Universal PCR master mix (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands), primers (see primers described above) and MGB-probes (Applied Biosystems) and a Taqman ABI Prism 7700 Sequence Detection System (Applied Biosystems). Used probes were 5'-FAM-TCTGCTGAT**C**AATCT -3' and 5'-VIC- TCTGCTGAT**G**AATCT - 3' (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions using

an annealing temperature of 60° C and optimized concentrations for primers and probes of 400 nmol/L and 50 nmol/L, respectively. We re-analysed genotypes in 18 samples by PCR-RFLP analysis using the BclI restriction enzyme and a digestion of 1 hour at 37 °C and found identical genotypes. The extracted DNA of the second study group of 1963 subjects was used to amplify the polymorphic region with PCR, which was carried out in a 10 μl reaction volume containing 5 ng of genomic DNA, 1.5 mM magnesium chloride, 0.2 mM of each deoxy-NTP, 200 nM of each primer (see above), 0.1 unit of Taq polymerase (Promega) and 10x PCR buffer (Promega). The PCR reactions were performed in a 384-wells thermocycler (MJ Research Tetrad). The genotypes were detected by the Single Base Extension (SBE) procedure using the following SBE primer: 5'- TTTTTTTTTTAAAGTAGACAAGTTATGTCTGCTGAT-3'. The SBE reactions were performed according to details provided by the manufacturer (ABI Prism® SnaPshot™ Multiplex Kit) with slight modifications. The genotypes thus generated were analyzed with the software program Genotyper 3.7 (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands) and also checked by eye. To confirm the accuracy of the genotyping, 150 randomly selected samples were genotyped for a second time with the same method. No discrepancies were found.

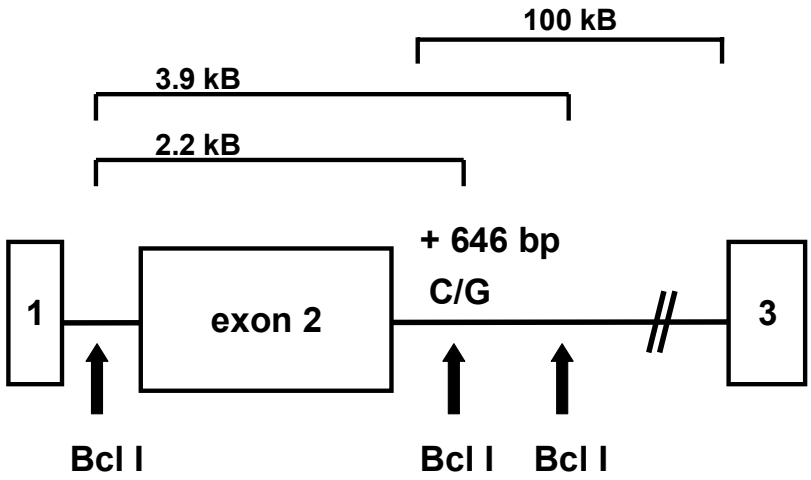

Figure 1: *First three exons of the Glucocorticoid Receptor gene. Localization of the BclI-restriction sites. The position of the variable BclI restriction site is indicated by C/G, 646 bp downstream from exon 2. Bp, basepairs.*

Statistical analysis Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, IL). Logarithmic transformations were applied to normalize variables if necessary and to minimize the influence of outliers. Association of the G-allele of the *BclI* polymorphism with

continuous variables was tested by linear regression analysis and adjusted for age and, if necessary, for sex and BMI. Bonferroni post hoc tests were used to test for differences between each genotype and to correct for multiple comparisons. To test for differences between categorized variables and genotypes Pearson Chi square test was used. Results are reported as means ± SE. *P* values are two-sided throughout, and a $p < 0.05$ was considered to indicate a significant difference.

Results

Identification of BclI polymorphism

We have identified a previously described *BclI* restriction fragment length polymorphism as a C/G single nucleotide polymorphism in intron 2 of the *GR* gene, 646 nucleotides downstream from exon 2 (figure 1). In previous studies [6, 9-12, 16], the *BclI* RFLP was detected by Southern blotting of *BclI* -digested total genomic DNA. The RFLP was assumed to be caused by a polymorphic *BclI* -site in intron 2 of the *GR g*ene [6, 7]. From the published sequence (GenBank NT_030707) we identified three *BclI* sites around exon 2 of the *GR* gene, located 392 bp upstream of exon 2, 646 bp downstream of exon 2 and 2301 bp downsteam of exon 2, respectively. PCR-RFLP and sequence analysis showed that the second site (646 bp downstream of exon 2) was indeed polymorphic (TGAT**C**A → TGAT**G**A), and we observed allelic frequencies similar to those reported previously for the *BclI* polymorphism. The fragment sizes expected (3.9 kb and 2.2 kb) were slightly different from those reported in the literature (4.5 kb and 2.3 kb) [7].

Functional studies in vivo

In study group 1, we found 79 CC-carriers (41.4 %), 91 CG-carriers (47.6 %) and 21 GG-carriers (11.0 %). Sexes were equally represented in the three genotype groups. Genotype distributions (Table 1) did not differ from those expected under Hardy-Weinberg equilibrium (HWE) conditions. Mean age and BMI of this subgroup are shown in table 1. At the second examination after 2.5 years, 58 of the 143 participants were homozygous C-allele carriers, 68 were heterozygous and 17 were homozygous for the G-allele. Also in these groups sexes were equally represented.

At baseline no differences in the early morning serum cortisol concentrations were found between genotype groups (p=0.31). However, after administration of 1 mg DEX, cortisol concentrations were lower in an allele-dosage way in heterozygous and homozygous G-allele carriers (p=0.18 and p=0.01, respectively), when compared to CC-carriers (p_{trend}=0.011, figure 2 A). The actual DEX concentrations did not differ in the three groups (p=0.69), so the differences in response to cortisol were not due to differences in DEX concentrations or metabolism.

Table 1: Description of the study populations

Study group (N)	1 (191)		2 (1963)		3 (370)	
	Mean	SE	Mean	SE	Mean	SE
Age (yrs)	66.9	0.4	67.2	0.2	77.8	0.2
Males/ Females	92/ 99		933/ 1030		370/ 0	
BMI (kg/m2)	26.2	0.3	25.8	0.1	25.4	0.2
SBP (mmHg)	139.4	1.4	137.6	0.5	156.3	1.2
DBP (mmHg)	74.9	0.7	73.7	0.3	83.9	0.6
Allele frequenties						
C-allele	65 %		62 %		67 %	
G-allele	35 %		38 %		33 %	

SE, Standard Error of the mean, BMI, body mass index, SBP, systolic bloodpressure, DBP, diastolic bloodpressure.

Two and half years later a low dose DST was performed and again there were no differences in fasting cortisol concentrations (p=0.39). The cortisol concentrations after the administration of 0.25 mg DEX, however, showed a same allele-dosage effect as after 1 mg DEX (p_{trend} = 0.017, figure 2 B). Heterozygous CG-carriers, as well as the GG-carriers had lower postDEX levels when compared to CC-carriers (p=0.032 and p=0.055, respectively). Again, these differences were not due to differences in DEX concentrations (p=0.96), so this suggests that G-allele carriers are more sensitive to DEX. We did not find any differences in fasting insulin concentrations between the genotypes (data not shown).

Anthropometry in elderly men and women

Study group 2 consisted of 364 male and 392 female homozygous C-allele carriers (CC), 435 male and 502 female heterozygous G-carriers (CG) and 134 male and 136 female homozygous G-allele carriers (GG). Genotype distributions did not differ from those expected under HWE conditions. As shown in Table 2, there were no significant age differences between the three genotype groups.Figure 3A shows that the G-allele is associated with a lower BMI (P_{trend}= 0.006). Homozygous G-allele carriers have a lower mean BMI compared to CG-carriers (P = 0.031) and to CC-carriers (P = 0.006). The WHR was also significantly lower in GG-carriers when compared to CG-carriers (P= 0.022) and CC-carriers (P = 0.049). Additional correction for smoking as a potential confounder did not change these results. No significant differences were found in height and systolic or diastolic blood pressure (Table 2).

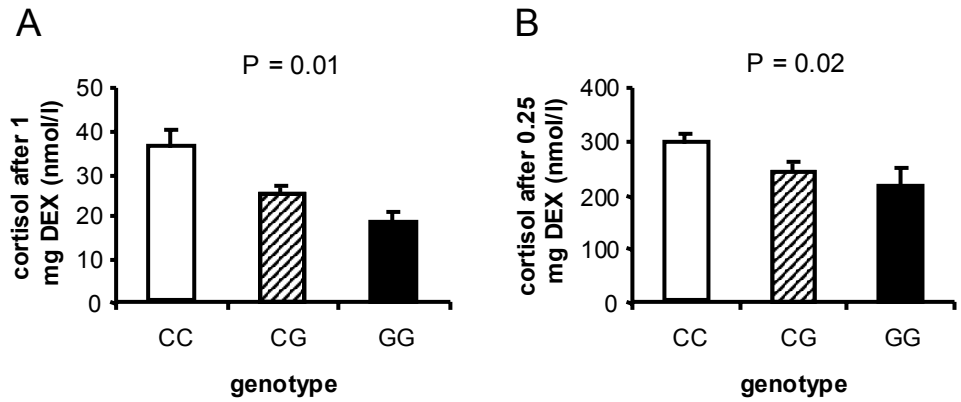

Figure 2: *Cortisol concentrations after administration of 1 mg DEX (A) and 2.5 years later after 0.25 mg DEX (B) in CC-carriers (white bars), CG-carriers (striped bars), GG-carriers (black bars). In both tests post-DEX cortisol levels were significantly lower in G-allele carriers*

BclI polymorphism in relation to Body composition

In study group 3 we found 165 homozygous C-allele carriers, 165 heterozygous G-allele carriers, and 40 homozygous G-allele carriers, which was compatible with the HWE. No significant age differences were present (table 3). Figure 3B shows again that the G-allele was associated with a lower BMI (P_{trend}= 0.05). No differences were found in height, total fat mass, trunk fat or WHR (Table 3). However, lean body mass, which we also corrected for height, tended to be lower in heterozygous and homozygous G-allele carriers (P_{trend}= 0.07). BMI and lean mass remained lower in G-allele carriers after an additional correction for smoking.

Table 2: Age, anthropometric parameters and blood pressures in the three genotypes in study group 2

Genotype (N)	CC (756)		CG (937)		GG (270)		
	Mean	SE	Mean	SE	Mean	SE	P
Age (yrs)	67.2	0.3	67.3	0.2	67.0	0.4	0.78
Height (m)	168.2	0.3	167.8	0.3	168.7	0.6	0.66
WHR	0.91	0.00	0.91	0.00	0.89	0.01	0.08
SBP (mmHg)	137.6	0.8	137.5	0.7	137.7	1.3	0.78
DBP (mmHg)	73.8	0.4	73.5	0.4	74.4	0.7	0.25

All parameters were adjusted for age and sex, and tested using linear regression analysis. Blood pressures were also adjusted for BMI. SE, Standard Error of the mean, WHR, waist to hip ratio, SBP, systolic bloodpressure, DBP, diastolic bloodpressure.

BclI polymorphism in relation to Bone Mineral Density

In study group 2 bone mineral density was also measured. In females (n= 1030), BMD in the lumbar spine (CC: 1.03 ± 0.01, CG: 1.01± 0.01, GG: 0.98 ± 0.01, P_{trend} =0.019), as well as in the trochanter (CC: 0.72 ± 0.01, CG: 0.71± 0.01, GG: 0.69 ± 0.01, P_{trend} =0.062) and the femoral neck (CC: 0.81 ± 0.01, CG: 0.80 ± 0.01, GG: 0.78 ± 0.01, P_{trend} =0.184) was lower in an allele-dosage way in G-allele carriers. After an additional correction for BMI, only in the lumbar spine a tendency towards lower BMD in G-allele carriers remained (L_2-L_4: p=0.066, trochanter: p=0.364 and femoral neck: p=0.523).

Table 3: Age, anthropometric parameters and body composition by *BclI* genotype in study group 3

Genotype (N)	CC (165)		CG (165)		GG (40)		
	Mean	SE	Mean	SE	Mean	SE	P
Age (yrs)	77.8	0.3	77.9	0.3	77.6	0.6	0.81
Height (m)	1.73	0.5	1.72	0.5	1.73	0.9	0.79
WHR	0.98	0.00	0.98	0.00	0.99	0.01	0.38
Fat mass (kg)	21.5	0.5	20.4	0.4	21.0	0.8	0.24
Trunk fat (kg)	10.8	0.2	10.3	0.2	10.8	0.4	0.25

Test for differences between the three genotypes. All parameters were adjusted for age. SE, Standard Error of the mean, BMI, body mass index, WHR, waist to hip ratio.

In males, a significant interaction between age and BMD existed (p<0.05), therefore we analyzed them in 2 age groups based on the median age. In the older age group (age 67-80 yrs) no differences between the 3 genotypes were found. However, in the younger age group (age 55-67 yrs), homozygous G-allele carriers had lower BMD in the femoral neck (CC: 0.89 ± 0.12, CG: 0.88 ± 0.13, GG: 0.86 ± 0.12, P_{trend} =0.044) and in the trochanter (CC: 0.86 ± 0.13, CG: 0.86 ± 0.13, GG: 0.82 ± 0.12, P_{trend} =0.026). After an additional correction for BMI, the differences in BMD in femoral neck and trochanter were no longer statistically significant (p=0.133 and p=0.117, respectively). No differences were found in BMD in lumbar spine.

Discussion

We identified the *BclI* restriction site polymorphism of the glucocorticoid receptor gene as a C/G single nucleotide polymorphism in intron 2, 646 nucleotides downstream of exon 2. This finding offered the possibility to use methods, which are less labour-intensive than Southern blotting to genotype and, thus, facilitate screening of large groups. The lengths of the restriction fragment

sequences were 2.2 and 3.9 kb for the shorter and the larger allele, slightly different from the previously described 2.3 and 4.5 kb restriction fragments [7], possibly due to the relatively accurate sizes as determined by agarose gel electrophoresis. According to the common nomenclature the polymorphism could be named IVS2+646, however in view of the existing literature on this polymorphism we chose to retain the currently used name '*Bcl*I polymorphism'.

Carriers of the G-allele had lower cortisol levels after both 1 mg and 0.25 mg DEX, suggesting that they are more sensitive to the feedback action of GCs on the HPA-axis. In accordance, Panarelli et al [10] found in a group of 64 men 6 homozygous carriers of the 4.5 kb allele (G-allele) who had increased skin vasocontriction in reaction to budesonide compared to 7 homozygous carriers of the 2.3 kb allele (C-allele), which also suggests hypersensitivity to GCs. In addition, in a study of 284 Swedish men, stimulated cortisol secretion after a standardized lunch differed between the *Bcl*I genotypes, which suggests an association between the *Bcl*I polymorphism and regulation of the HPA-axis as well [6]. However, we cannot explain the negative results after a 0.5 mg DST described in the same report. In another study, the *Bcl*I polymorphism was found to be associated with hyperinsulinaemia and relative insulin resistance in obese women [11]. In contrast, we did not find an association with insulin levels. This might be due to the fact that we studied a normal weight population and not an obese population.

In our population-based study in elderly subjects (study group 2), we found an association between the G-allele and lower BMI and WHR. We confirmed this association of the G-allele with lower BMI in a group of elderly males (study group 3). Our findings are in contrast with several previous reports. Rosmond et al. reported that the G-allele was associated with *increased* abdominal sagittal diameter, BMI, WHR and leptin levels [6]. In two other studies, also an association between the G-allele and increased abdominal fat mass was found, but no relation with BMI or total fat mass [9, 16]. We can only speculate what causes the lower BMI we observed in heterozygous G-allele carriers, and homozygous G-allele carriers in particular. As the total body fat mass was equal in the three genotypes, this lower BMI could be explained by a lower lean body mass, as was indeed observed in elderly males (study group 3), although this was only a trend. One of the main differences between earlier studies and ours is the age of the subjects: we studied older populations. We speculate therefore that the differences in this population are caused by a life long exposure to the increased GC sensitivity associated with the G-variant. It is possible that the influence of GCs on the normal changes in body composition that accompany ageing, results in G-allele carriers in an additional loss of lean mass. In this context, it is known that most obese individuals have an increased lean body mass, as well as fat mass. However, in patients suffering from Cushing's syndrome, the obesity is not accompanied by an increase, but by a decrease in lean body mass [17]

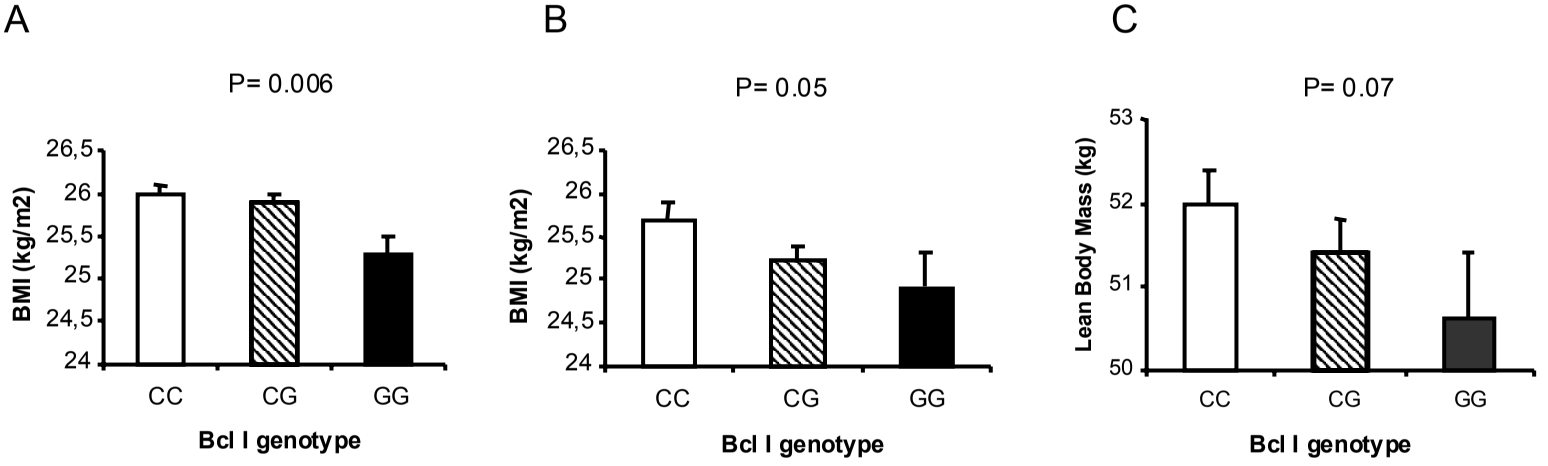

Figure 3: *The relationship between BMI and BclI genotype in (A) study group 2 (mean age 67.2 ± 0.2) and in (B) study group 3 (mean age 77.8 ± 0.2), and the relationship between lean body mass and BclI genotype in study group 3 (C). White bars: CC-carriers, striped bars: CG-carriers, and black bars: GG-carriers.*

In parallel to this, G-allele carriers could have lower lean mass due to a subtle, but life long exposure to the catabolic effect of hypersensitivity to GCs, instead of exposure to high cortisol levels.

GCs are also known to decrease bone mineral density [18]. In addition, a lower body weight is accompanied by lower BMD [19]. Thus, we would have expected to find lower BMD in the G-allele carriers. In our population-based study in the elderly we found a subtle negative effect, in a dosage-allele way, of the BclI polymorphism on BMD in females, but only in the youngest half (55-67 yrs) of the males in this population. Most of these effects can probably be explained by the differences in BMI, which we found to be lower in the G-allele carriers. Moreover, statistical significance for differences in BMD disappeared after an additional correction for BMI. We previously described in the same population (study group 1) a polymorphism in codon 363 of the GR gene, which was also associated with increased GC sensitivity with respect to the negative feedback mechanism [3]. For this N363S polymorphism we only found differences in cortisol levels between genotypes after 0.25 mg DEX, but not after 1 mg DEX, while for the BclI genotypes differences were present in an allele-dosage way after both 1 mg and 0.25 mg DEX. In this population the allele frequency of the G-variant of the BclI polymorphism is much higher (35 %) than the S-allele of the N363S polymorphism (3%), thus the statistical power to detect differences between N363S genotypes was less.

In contrast, the N363S polymorphism was associated with higher BMI in the elderly [3]. We speculate that the N363S polymorphism predominantly affects fat mass, whereas the BclI polymorphism has an effect on lean body mass, possibly due to a tissue specific regulation of the expression of the GR gene. In this context, Panarelli et al [10] demonstrated increased sensitivity to GCs in fibroblasts in vivo, while in vitro experiments on leucocytes showed a tendency towards decreased sensitivity to DEX, so these contrasting findings suggest that tissue-specificity might play a role in the associations observed for the BclI polymorphism.

At present, we do not know the exact mechanism through which the BclI polymorphism exerts its effects. There is no obvious function in processing of GR pre-mRNA. Possibly this polymorphism is linked to variations in the promoter region (increased expression) or 3`-UTR (increased stability) of the GR gene. We found no linkage with the previously described [2] polymorphisms in codons 363 or 22/23 (data not shown). A less likely possibility is linkage to one or more genes in the vicinity of the GR gene. However, the choice is rather limited since, in view of our results, this other gene would have to play a direct role in the sensitivity of the HPA-axis. Other mechanisms leading to altered GC sensitivity also exist. It is known, that locally GR sensitivity can be influenced by cytokines, as reported in asthma [20]. Also, alternative splicing could play a role, however, to our knowledge the polymorphic BclI site is not located in the vicinity of a sequence involved in the splicing process.

In conclusion, we identified an *Bcl*I-RFLP of the *GR* gene as a C to G polymorphism in intron 2, which increases the sensitivity of negative feedback mechanism of GCs. Furthermore,

we found an association between the presence of the G-allele and lower BMI in two different healthy older populations, as well as a tendency towards a lower lean body mass in older males. The exact mechanism of the effects of this RFLP is not clear, and needs further investigation.

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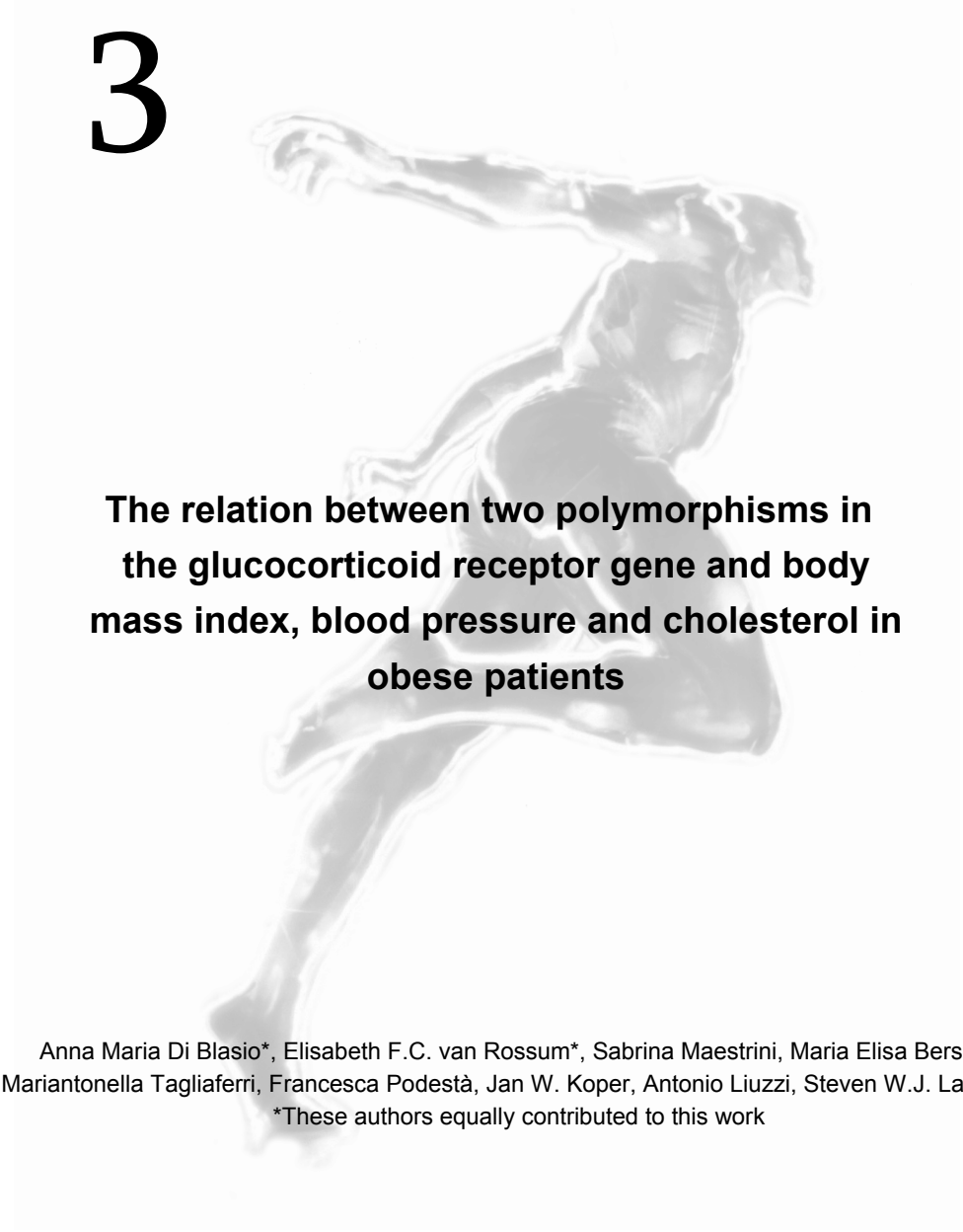

The relation between two polymorphisms in the glucocorticoid receptor gene and body mass index, blood pressure and cholesterol in obese patients

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Abstract

Objective: We have recently reported that, in healthy elderly Dutch individuals, a N363S polymorphism in the glucocorticoid receptor (GR) gene is associated with higher sensitivity to low dose dexamethasone (0.25 mg), evaluated as both cortisol suppression and insulin response, and with an increased body mass index (BMI). In the present study we investigated the role of the N363S polymorphism, and a BclI restriction site polymorphism in a group of Italian patients with severe obesity. Design: Two hundred and seventy-nine patients (mean BMI 45.9 ± 0.9 Kg/m^2) were genotyped using both PCR-restriction fragment length polymorphism analysis and Taqman Sequence Detection System. Determination of several metabolic and antropometric parameters was also performed in order to correlate them to the genotype.
Results: In this group of obese patients, 13 subjects (8 females and 5 males) were heterozygous for the N363S variant (allelic frequency 2.3%) and had significantly higher BMI ($p < 0.04$), resting energy expenditure ($p < 0.03$) and food intake ($p < 0.01$) when compared to wild-type homozygotes. When the data were analysed according to sex, female heterozygotes for the N363S allele had significantly higher BMI ($p = 0.04$), resting energy expenditure ($p = 0.03$) and food intake ($p = 0.008$) than obese women with the wild type 363 GR gene. Male carriers of this variant also had higher values for these variables although the differences did not reach statistical significance. A case control study with homozygous wild type obese subjects which were age-, sex- and BMI-matched, revealed no difference in resting energy expenditure and food intake. The allele frequency of the BclI variant was 27% (89 females and 41 males out of 269 subjects). No differences in anthropometric and metabolic parameters were found between subjects heterozygous or homozygous for this variant GR in this obese population. However, when we studied the effect of the presence of the BclI polymorphism and the N363S variant in the same individual, we found that the subjects who carried both polymorphisms had a tendency towards higher systolic and diastolic blood pressure and significantly higher total and LDL-cholesterol levels ($p=0.005$ and $p=0.05$, respectively).
Discussion: Taken together the results of this study and those obtained in the Dutch population, we speculate that heterozygous carriers of the N363S variant who develop obesity, may become even more obese, possibly because they have a hypersensitive insulin response and thus, via activation of lipogenesis, store fat more efficiently. Furthermore, these data suggest that N363S carriers who carry the BclI polymorphism as well, tend to have a slightly unfavourable cardiovascular profile.

Introduction

There is a considerable variability in the sensitivity to glucocorticoids across individuals [1]. Some of these differences have been correlated to polymorphisms in the glucocorticoid receptor (GR) gene [2.] One of the five polymorphisms identified, is an A to G substitution in codon 363 in exon 2 of the *GR*-gene causing a change of asparagine to serine in the GR protein.
This N363S polymorphism was, in the first reported study, associated with higher sensitivity to low-dose (0.25 mg) dexamethasone, with respect to both cortisol suppression and insulin response (1). In a group of 216 elderly individuals from the Rotterdam Study, 12 heterozygous N363S carriers tended to have a higher BMI (28.1 ±1.09 vs 26.6 ± 0.26 Kg/m^2; p<0.07); this was confirmed two and half years later in a second study in which 161 of these same individuals participated : 8 N363S heterozygous carriers vs 153 controls demonstrated a significantly higher BMI (28.3 ± 1.52 vs 25.5 ± 0.3 Kg/m^2; p<0.05).
In the subsequent years a number of investigators have reported on the association between the N363S polymorphism and obesity with contradictory results. Lin and coworkers[3] reported a highly significant association with BMI in a cohort of Australian subjects of British descendent. The overall penetrance in participants with the N363S variant was 83% in overweight normotensive individuals and 100% in overweight hypertensive subjects. However, a Swedish study[4] did not confirm this association with BMI, while in an English study[5] only a significant increase of waist-hip ratio (WHR) was observed in male heterozygotes for the N363S variant.
It is noteworthy that most subjects heterozygous for the N363S variant should be considered overweight rather than obese as, in the populations studied, the mean BMI reported never exceeded 28 Kg/m^2**.** To further investigate the linkage between the N363S polymorphism and obesity, we studied a series of Italian patients with severe obesity (mean BMI of 45.9±0.9 Kg/m^2) and evaluated whether the N363S allele is associated with any subphenotypic characteristics of obesity.

Recently, it has been shown that a very common *BclI* restriction site polymorphism of the GR gene is associated with abdominal obesity and higher systolic blood pressure[6,7]. In the present study we also determined the effect of this *BclI* polymorphism on anthropometric and metabolic parameters and the potential effects of the interaction between these two gene variants.

Materials and Methods

Patients

The present study included 279 consecutive obese patients (185 women, 94 men). Their mean age was 45.6± 0.9 years with a mean BMI of 45.9 ± 0.9 kg/m^2 (range 36-85). They were referred, between September 1998 and November 2000, to the Division of Endocrinology and

Metabolic Diseases of the S. Giuseppe Hospital, Istituto Auxologico Italiano (Verbania) for medical problems related to obesity.

Twenty-seven (16 women and 11 men) out of the 279 patients had diabetes mellitus well-controlled by diet (HbA_1C= 6.8 $\pm$ 0.2%). All patients had normal thyroid function and none of them had concomitant severe renal, hepatic or cardiac disease. Body weight was stable for the last 4 weeks before admission.

The patients underwent a study protocol including evaluation of BMI, measurement of WHR, Resting Energy Expenditure (REE), energy intake, and determination of serum total cholesterol, high density lipoprotein (HDL)-cholesterol, triglycerides, fasting insulin and fasting glucose levels, as well as serum leptin concentrations.

WHR was calculated on the basis of the measurements taken at patients' admission. Blood sampling to determine leptin levels and other biochemical parameters, assessment of REE and body composition were performed after a 12 h-fast and before beginning treatment. Patients also underwent a 7-day dietary recall to estimate their usual daily energy intake. The study protocol was approved by the Institution Ethics Committee; the aim and the design of the study were explained to the patients who gave their informed consent. Genotype frequency of the patients was compared with that of 106 lean (BMI 22.4 + 0.5 kg/m^2) subjects (76 females and 30 males). These subjects were clinical and laboratory workers and medical students.

Measurements

REE was assessed by a computerized, open-circuit, indirect calorimetry system that measured resting oxygen uptake and resting carbon dioxide production using a ventilated canopy (Sensormedics, Milano, Italy). REE was measured at 08.00 h after an overnight fast, in a comfortable and thermo-regulated (22-24 C°) room where only the investigator and the patient were present. After a 10 minute period of steady-state, values were recorded each minute for 30 minutes; the mean value was then expressed as Kcal/24h.

Body fat distribution was estimated by WHR. The waist circumference was taken to the smallest standing horizontal circumference between ribs and the iliac crest, the hips circumference was taken as the largest standing horizontal circumference of the buttocks.

Glucose, total cholesterol, HDL cholesterol, triglycerides and insulin were measured by enzymatic methods (Boerhinger-Mannheim Kits, Germany,). Low density lipoprotein (LDL)-cholesterol was calculated using the following formula: LDL-cholesterol = total cholesterol - ((triglycerides/5) + HDL-cholesterol). HbA_1C (Boerhinger-Mannheim Kits, Germany) were determined by immunoenzymatic methods (Tosoh, Kyobashi Chuo-Ku, Tokio, Japan). FT4 and TSH were measured by radioimmunoassay (DPC Euro/DPC Ltd Llanberis,UK). Serum leptin levels were measured by radioimmunoassay using reagents supplied by Linco Research Inc (St Louis, MO, USA). In this assay, detection limit is 0.009 nmol/L; the intra-assay variation is 2.2% at 0.375 nmol/L, 2.7% at 1.56 nmol/L, and 5.9% at 3.92 nmol/L; inter-assay variation from 10

different runs of 3 serum samples is 4.3%, 4%, 6.9% at the concentration of 0.318, 1.31 and 3.5 nmol/mL respectively. In 32 lean subjects (BMI 18-25 Kg/m^2) reference limits (2.5-97.5 percentiles) were 0.061-0.323 nmol/L in men and 0.162-1.08 nmol/L in women.

Insulin sensitivity was evaluated by the Quantitative Insulin Sensitivity Check Index (QUICKI). This index derives from a mathematical model that takes into account the logarithm of fasting insulin and glucose levels to evaluate insulin sensitivity and has a very good linear correlation with the gold standard clamp measurement [8].

Genetic analysis

N363S polymorphism DNA was extracted from peripheral blood leukocytes using standard techniques. PCR amplification of the relevant fragment of the GR gene was carried out employing primer sequences and amplification conditions as described previously[2]. Restriction fragment length polymorphism analysis was carried out to determine genotypes. The PCR-products were digested with Tsp509I (New England Biolabs, Inc) at 65° C for 1 hour. Tsp509I cleaves at two restriction sites in the wild type (at 95 and 114 bp) and at one restriction site (at 95 bp) in the polymorphic variant. Fragment analysis was carried out with ethidium bromide on a 3% agarose-gel (MP-Boehringer, Mannheim).

BclI polymorphism DNA was genotyped by allelic discrimination using TaqMan Universal PCR master mix (Applied Biosystems, Branchburg, New Jersey, USA), primers and VIC- and FAM-labeled MGB-probes as previously described (van Rossum et al, 2003) and a Taqman ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60° C and optimized concentrations for primers and probes of 400 nmol/L and 50 nmol/L, respectively. We re-analysed genotypes in 18 samples by PCR-restriction fragment length polymorphism analysis using the *BclI* restriction enzyme for digestion at 37 °C of 1 hour and found identical genotypes.

Statistical Analysis

Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, IL). If appropriate logarithmic transformations were applied to normalize variables and to minimize the influence of outliers. The relationships between the GR polymorphisms and BMI as well as the other parameters were studied by one way analysis of variance (ANOVA) for each sex seperately. To investigate the effect of the *BclI* polymorphism on the N363S polymorphism we compared two groups of N363S-carriers: one group without the *BclI* polymorphism and a second group with carriers of both the N363S and at least one copy of the *BclI* variant. Because of the low numbers we analyzed these data for men and women together and adjusted for age and sex testing by ANCOVA using the general linear model procedure. Data are expressed as mean $\pm$ S.E.M. *P* values < 0.05 were considered to indicate a significant difference.

Results

N363S polymorphism

Genotype analysis of the 279 obese patients studied identified 13 heterozygotes for the N363S allele. In contrast, among the 106 lean subjects examined, only two heterozygotes were found. Thus, the N363S allelic frequency was 2.3% and 0.9 % in the obese and lean subjects, respectively. As previously observed in three other European populations, no N363S homozygotes were found [1,4,5]. In view of the frequency of the variant allele, this is not unexpected.

Table 1: Values (mean ± S.E.M.) of the demographic, anthropometric and metabolic parameters of the obese patients according to gender

Variable	**Men (94)**	**Women (185)**
Age (yr)	42.04 ± 1.5	47.5 ± 1.5
BMI (Kg/m^2)	46.1 ± 0.7	45.7 ± 0.5
WHR (cm)	0.98 ± 0.007	0.8 7± 0.006*
BP (sys)	136.3 ± 1.5	135.9 ± 1.2
BP (dia)	83.4 ± 0.9	82.6 ± 0.8
Energy intake (Kcal/day)	3728.2 ± 160	3181.5 ± 123.6†
REE (Kcal/day)	2290.7 ± 36	1799.4 ± 27.1*
Leptin (nmol/L)	1.97 ± 0.099	3.12 ± 0.081*
Total cholesterol (mmol/L)	5.45 ± 0.12	5.41± 0.08
LDL-cholesterol (mmol/L)	3.40 ± 0.10	3.36 ± 0.08*
HDL-cholesterol (mmol/L)	1.07 ± 0.02	1.33 ± 0.02*
TGL (mmol/L)	2.19 ± 0.19	1.6 ± 0.07†
FT4 (nmol/L)	163.4 ± 1.67	159.5 ± 2.5
TSH (mU/L)	2.2 ± 0.13	2.6+ ± 0.2
IRI (pmol/L)	131.3 ± 7.74	111.2 ± 5.74
QUICKI	0.321 ± 0.005	0.325 ± 0.003

* $P < 0.001$; women vs men † $P < 0.01$; women vs men. BMI, body mass index, WHR, waist-to-hip ratio, BP, blood pressure, REE, resting energy expenditure, HDL, high density lipoprotein, TGL, triglycerides, FT4, free thyroxine, TSH, thyroid stimulating hormone, IRI, immunoreactive insulin, QUICKI , Quantitative Insulin Sensitivity Check Index

The heterozygous subjects had significantly higher BMI ($p< 0.04$), REE ($p<0.03$) and food intake ($p<0.01$) when compared to homozygous wild-types. Table 1 presents a comparison of the demographic, anthropometric and metabolic parameters of the obese patients according to gender. Most parameters were strongly associated to sex. Thus, in view of the hormonal and physiological differences between males and females, the association of the GR genotype and the clinical and metabolic parameters considered were analyzed separately for the two sexes. Five of the 94 men and 8 of the 185 women were N363S heterozygotes with similar allelic frequencies (2.6% and 2.1%, respectively). As indicated in Table 2, women heterozygous for the N363S allele had significantly higher BMI ($p = 0.04$), REE ($p = 0.03$) and food intake ($p = 0.008$). Male carriers of this variant also had higher values for these variables although the differences did not reach statistical significance. As previously observed[1], fasting insulin levels in both male and female N363S carriers were not different from those of wild type subjects. Also the presence of the N363S polymorphism did not influence insulin sensitivity as the results of the QUICKI index were similar in the two groups. Moreover, thyroid function, as assessed by TSH and FT4 measurements, was also similar in the wild type and heterozygous subjects. Furthermore, the anthropometric data of the 13 N363S carriers were also compared with a similar group of wild-type obese subjects, carefully matched for age, sex and BMI. When this analysis was performed, food intake and REE values among the two groups were not significantly different (data not shown).

BclI polymorphism

The *BclI* allelic frequency observed in our obese patients was 73% and 27% for the C and G allele respectively. We did not find any differences between the homozygous C-allele carriers, heterozygous G-allele carriers and homozygous G-allele carriers in BMI, WHR, blood pressures, food intake, REE, leptin, lipids, thyroid hormones or insulin sensitivity parameters.

Interaction of the BclI polymorphism and N363S polymorphism

Seven out of the 13 heterozygotes for the N363S variant, also carried the *BclI* polymorphism (all heterozygotes). To investigate whether there is an effect of the presence of the *BclI* polymorphism on the N363S polymorphism we compared carriers of the N363S polymorphism and carriers of both N363S and *BclI* polymorphisms (only heterozygous G-allele carriers, because we did not find any homozygous G-allele carriers who had also the N363S variant). For this purpose, we analyzed the two sexes together and corrected for age and sex. As shown in table 3, carriers of both polymorphisms had a tendency towards higher systolic ($p=0.08$) and diastolic ($p=0.06$) blood pressure. BMI and WHR were not significantly different between the two groups. Total and LDL- cholesterol levels however, were higher ($p=0.005$ and $p=0.05$, respectively) in the group who carried both the N363S and the *BclI* polymorphism. No

differences were found in other parameters, such as insulin sensitivity parameters, food intake, leptin, HDL-cholesterol, triglycerides and thyroid hormones.

Table 2: Demographic, anthropometric and metabolic parameters of the obese patients according to the genotype

Sex (N)	**Men (94)**		**Women (185)**	
Genotype (N)	**wild type (89)**	**N363S (5)**	**Wild type (179)**	**N363S (8)**
Age (yr)	42.6 ± 1.6	41 ± 3.3	47.6 ± 1.2	45 ± 4.2
BMI (Kg/m^2)	46.1 ± 0.7	47.8 ± 1.5	45.5 ± 0.5	51.4 ± 3.6 †
WHR	0.98 ± 0.01	0.99 ± 0.02	0.86 ± 0.01	0.87 ± 0.03
BP (sys)	136 ± 1.6	141± 5.1	136 ± 1.3	133 ± 5
BP (dia)	83 ± 1.0	90 ± 1.5	82.5 ± 0.8	83.7 ± 2.6
Intake (Kcal/day)	3720 ± 160	4298 ± 1103	3103 ± 112	4496 ± 1107 *
REE (Kcal/day)	2286 ± 37.8	2475 ± 113	1787 ± 27.5	2045 ± 131†
Leptin (nmol/L)	2.01 ± 0.73	1.32 ± 0.16	3.08 ± 0.08	3.66 ± 0.49
Total chol (mmol/L)	5.53 ± 0.12	4.63 ± 0.26	5.41 ± 0.08	5.43 ± 0.58
LDL (mmol/L)	3.46 ± 0.10	2.42 ± 0.39	3.35 ± 0.08	3.49 ± 0.47
HDL (mmol/L)	1.08 ± 0.02	0.87 ± 0.07	1.33 ± 0.03	1.36 ± 0.09
TGL (mmol/L)	2.18 ± 0.2	2.93 ± 0.77	1.62 ± 0.07	1.31 ± 0.18
FT4 (nmol/L)	163.4 ± 3.9	157 ± 1.2	159.5 ± 2.6	155 ± 3.6
TSH (mU/L)	2.2 ± 0.1	2.5 ± 0.8	2.6 ± 0.2	2.3 ± 0.5
IRI (pmol/L)	132 ± 7.89	114 ± 12.9	110 ± 5.7	121 ± 22.2
QUICKI	0.32± 0.01	0.31 ± 0.00	0.33 ± 0.00	0.32 ± 0.01

* $P < 0.01$ † $P < 0.05$, BMI, body mass index, WHR, waist-to-hip ratio, BP, blood pressure, REE, resting energy expenditure, chol, cholesterol, HDL, high density lipoprotein, TGL, triglycerides, FT4, free thyroxine, TSH, thyroid stimulating hormone, IRI, immunoreactive insulin, QUICKI , Quantitative Insulin Sensitivity Check Index

Table 3: BMI, blood pressures and cholesterol of the female and male N363S carriers and carriers of both N363S and *BclI* polymorphism

Genotype (N)	**N363S carriers (5)**	**N363S + BclI carriers (7)**
BMI (Kg/m^2)	48.1 ± 1.7	51.8 ± 4.0
BP (sys)	131 ± 7.8	139 ± 3.5
BP (dia)	84 ± 4.0	87 ± 1.8
Total cholesterol (mmol/L)	4.25 ± 0.77	5.88 ± 0.24 *
LDL-cholesterol (mmol/L)	2.20 ± 0.59	3.80 ± 0.26 †

* P = 0.005; N363S carriers vs N363S + BclI carriers.† P = 0.05; N363S carriers vs N363S + BclI carriers. BMI, body mass index, BP, blood pressure, LDL, low density lipoprotein

Discussion

We have previously demonstrated that in a normal elderly population a polymorphism in exon 2 of the GR gene, leading to an asparagine to serine change at codon 363, is associated with an increased sensitivity to glucocorticoids and a marginal increase of BMI[1]. In the present study, we evaluated a cohort of Italian severely obese patients in order to verify whether this genetic variant is correlated to any of the anthropometric, metabolic and clinical features of obesity. Out of 279 obese subjects studied, 13 were found to be heterozygous for the N363S allele**.** Due to the known hormonal and metabolic differences between men and women, we analyzed the two groups separately and found that female carriers of the N363S variant (n=8) had a significant increased BMI, food intake and REE. These same variables were also increased in heterozygous men (n=5), but the differences did not reach statistical significance.

The patients were also studied for the presence of a *BclI* RFLP of the GR gene that has been recently shown to be associated with abdominal obesity [6,7] The allelic frequency of this *BclI* polymorphism in our obese patients was 27%. We did not find differences between noncarriers and carriers of this *BclI* polymorphism in our obese patients. These data are in contrast with the results of the studies mentioned above. One of the explanations for this discrepancy could be related to the characteristics of the different populations studied. Indeed, the subjects included in this study were selected for the presence of severe obesity and thus it can be expected that differences in anthropometric parameters will only be found when there is a strong effect of the polymorphism.

Because 7 out of 13 N363S carriers were also heterozygous for the G-allele of the *BclI* polymorphism we were interested in the interaction of these variations in the GR gene. Carriers of both polymorphisms tended to have higher systolic and diastolic blood pressure and higher total and LDL-cholesterol.

We do not know the mechanism underlying the observed differences between N363S carriers and N363S + *BclI* carriers. It is also unknown how the *BclI* polymorphism exerts its effect. It is possible that it directly affects GR gene expression or considering its intronic location- is linked to a locus in one of the exons or the promoter region.

It is well known that in obese subjects energy expenditure and food intake are directly correlated with BMI [9,10]. In order to gain more insight into the physiological significance of our findings, we performed a case-control evaluation in which the 8 N363S heterozygous female carriers were compared with a similar number of age-, sex-, and BMI matched obese women with the wild type GR gene. The results of this analysis indicated that the differences in food intake and energy expenditure were not significant anymore. These observations suggest that the effect of the N363S variant on obesity results primarily in an increased BMI. In our original study[1], we documented that heterozygotes for the N363S polymorphism have greater insulin response to dexamethasone administration than non-carriers. Thus, we speculate that one of the patho-physiological mechanisms underlying the observed phenomenon could be a relative hypersensitivity for insulin elicited by changes in serum cortisol levels that, theoretically, could result in increased lipogenesis and eventually in an increased BMI. While it is clear that environmental factors play a considerable role in the development of obesity, a genetic influence on adipose tissue accumulation and distribution has also been demonstrated, with estimation of an additive genetic contribution to the expression of some obesity-related traits of 50% or greater [11,12]. The search for genes underlying normal variation in human obesity has so far being challenging. Some efforts have been focused on the identification of rare single gene defects that typically lead to extreme obesity [13,14]. In addition, quantitative trait locus mapping methods for complex phenotypes have been used with some success [15]. In the present study we analyzed the potential influence of a polymorphic single gene abnormality in codon 363 of the GR gene, which seems to have a small effect in the normal population, but exerts an adiposity-enhancing effect in obese individuals.

Since our first report, several population studies have been performed to look for associations between the presence of the N363S variant and long-term effects on BMI, glucose metabolism and hypertension. In a group of 109 women with the polycystic ovary syndrome, the N363S variant was not related to patients with and without androgen excess [16]. Rosmond [4] did not find any association between the N363S variant and obesity, cortisol secretion (including the response to 0.5 mg dexamethasone), blood pressure and insulin sensitivity, while Dobson et al. only observed an increased WHR in male N363S carriers [5]. In contrast, Lin et al found ~~a~~ 9.4% heterozygotes and ~~a~~ 2% homozygotes in a white Australian population of British

descent[3]. Apart from a highly significant association with BMI (heterozygotes) and obesity (homozygotes), they extended their studies reporting that this genetic variant also plays a role in type 2 diabetes mellitus and coronary artery disease [17].

The contradictory results in the studies mentioned above, with regard to an association between N363S heterozygosity and BMI reflects the problems which are inherent to the study of low frequency genetic variants: differences in the study population, in the measuring of the various forms of obesity, insulin sensitivity and dexamethasone sensitivity underline the difficulties of studying polymorphic single gene variations which exert a small effect [12]. This is further underlined by the observation that the N363S variant in the GR gene was not detected in any of 192 Japanese individuals [18]. As we have shown in the present study the presence of two polymorphisms which previously both have been associated with increased BMI or abdominal obesity can have a combined effect. Thus, not only differences in frequency of the N363S variant are important for explaining the contrasting findings in different ethnic populations, but also the frequency of the *BclI* polymorphism and in particular the combination of the two polymorphisms in the same individuals can play a role.

In conclusion, we demonstrated that patients with severe obesity, who are heterozygous for the N363S variant of the GR gene have a significantly higher BMI than obese patients with the wild type GR gene. Furthermore, N363S carriers who carried the *BclI* polymorphism as well, tended to have higher blood pressures and serum cholesterol levels.

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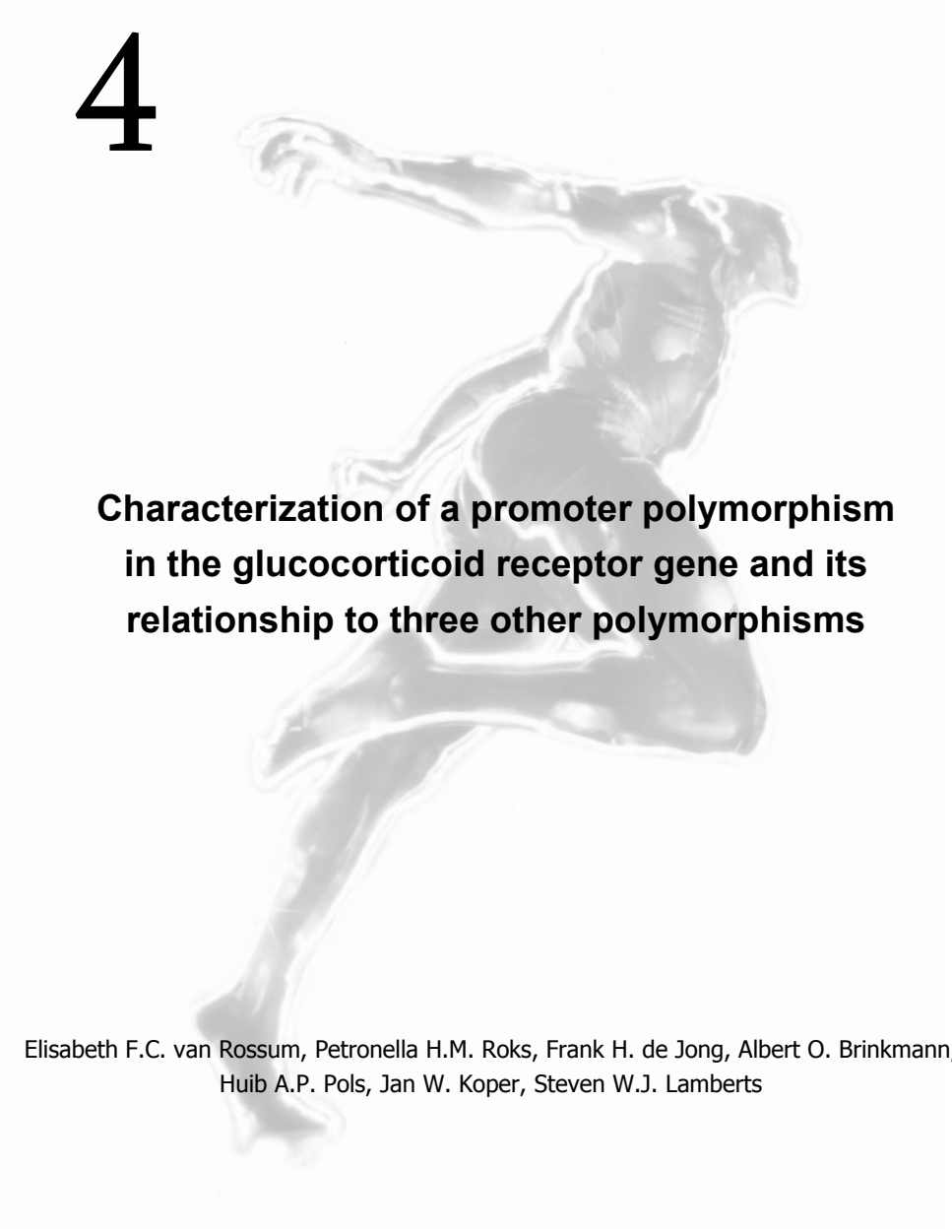

Characterization of a promoter polymorphism in the glucocorticoid receptor gene and its relationship to three other polymorphisms

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Abstract

Objective: sensitivity to glucocorticoids within the normal population is highly variable and partly determined by polymorphisms in the glucocorticoid receptor (GR) gene (NR3C1). We investigated the exact sequence alteration of a *TthIII*I polymorphism in the *GR* gene, whether it is associated with glucocorticoid sensitivity, and its relationship to 3 polymorphisms of the *GR* gene (N363S, *Bcl*I, ER22/23EK). Design: two dexamethasone (DEX) suppression tests were performed with 1 and 0.25 mg DEX, respectively. Patients: we genotyped a random subgroup of 209 participants of the Rotterdam Study, a population-based study in the elderly. Measurements: anthropometric parameters, cortisol, insulin and glucose levels, and lipid concentrations were measured. Results: We identified the *TthIII*I polymorphism as a C to T mutation, 3807 bp upstream from the mRNA start site. We found 39.7 % CC-carriers, 44.5 % CT-carriers, and 15.8 % TT-carriers. No differences were found between *TthIII*I genotypes in sensitivity to DEX, baseline cortisol, insulin, glucose or cholesterol levels, or in anthropometric variables. However, all ER22/23EK-carriers also carried the *TthIII*I T-allele, and carriers of both these polymorphisms had a significantly smaller cortisol suppression after 1 mg DEX, lower fasting insulin levels, and lower total and LDL-cholesterol levels than *TthIII*I T carriers without the ER22/23EK variant and noncarriers. No interaction was found between the *TthIII*I variant and N363S or *Bcl*I polymorphisms. Conclusions: the *TthIII*I polymorphism is not functional by itself. However, the ER22/23EK polymorphism is without exception linked to the *TthIII*I T polymorphism and this haplotype is associated with a relative resistance to glucocorticoids, and a healthy metabolic profile.

Introduction

Glucocorticoids (GCs) are important in the regulation of numerous processes throughout the human body. Their effects are established by binding to the glucocorticoid receptor (GR) [1]. Variation in sensitivity to GCs within the normal population can be influenced by polymorphisms in the *GR* gene (NR3C1). Several of these polymorphisms have been reported. A polymorphism in codon 363 causes an amino acid change from asparagine (N) to serine (S), and has been shown to be associated with hypersensitivity to GCs, as well as an increased insulin response to GCs [2]. In some studies this polymorphism was associated with a higher body mass index (BMI) [2-6] or higher waist-to-hip ratio [7], while others showed no association with BMI [8-10]. Lin et al recently reported an association between the N363S polymorphism and coronary artery disease and several cardiovascular risk factors independent of weight [11].

A frequent *BclI* restriction site polymorphism was reported to be associated with abdominal obesity [12-14], increased insulin levels in obese women [15], and tissue specific differences in GC sensitivity [16]. Recently, we identified the exact mutation of this polymorphic site (C to G in intron 2), and showed an association between the G-allele of the *BclI* polymorphism and hypersensitivity to GCs in an elderly Dutch population [17]. In a larger group of Dutch elderly we found a lower BMI in heterozygous and homozygous G-allele carriers. This was confirmed in another group of Dutch elderly men [17]. In these males we also found a tendency towards lower lean body mass in G-allele carriers, which is in line with an increased sensitivity to GCs and possibly explains the lower BMI at older age.

A third polymorphism consists of two linked point mutations in codon 22 (silent mutation, changing codon 22 from GAG to GAA, both coding for glutamic acid (E)) and in codon 23 (changes from AGG to AAG, resulting in an amino acid change from arginine (R) to lysine (K)) [18]. We also studied this polymorphism in the elderly and showed an association with a relative resistance to GCs, better insulin sensitivity, as well as lower total and low-density lipoprotein (LDL)-cholesterol levels [19]. In another population of elderly men, again ER22/23EK-carriers tended to have lower total and LDL-cholesterol levels, and C-reactive protein (CRP) levels were significantly lower as well, which possibly reflects their better cardiovascular status [20]. Moreover, in this population the ER22/23EK variant was associated with longevity.

Previously, a polymorphic *TthIIII* restriction site in the promoter region of the GR gene has been described [21], which was associated with an increased basal cortisol secretion [22]. In the present study we investigated the exact sequence alteration of the *TthIIII* polymorphism and whether it is associated with altered sensitivity to GCs. We also studied the relationship between this promoter polymorphism and the N363S, ER22/23EK and *BclI* polymorphisms, as well as the clinical relevance of carrying more than one of these polymorphisms.

Materials and Methods

Subjects A total of 216 persons participated in the study, all participants in the Rotterdam Study (The Netherlands), who were at random selected from a population-based cohort study (7983 subjects) of the determinants of chronic disabling diseases in the elderly, as described previously [2].

Subjects with acute, psychiatric or endocrine diseases, including diabetes mellitus treated with medication, were not invited. Compared to all participants of the Rotterdam study, there were no differences in age and gender distribution and cardiovascular risk factors. The subjects gave their written consent to participate in the study, which received the approval of the Medical Ethics Committee of the Erasmus University Medical School.

Due to technical reasons genotype data on the *TthIII*I polymorphism were available of 209 persons. Their age varied between 53 and 82 years (99 men and 110 women with mean ages of 67.7 ± 0.6 and 66.0 ± 0.6 years, respectively). Genotype data of all four polymorphisms were available in a total of 181 subjects.

Anthropometric Measurements Body weight, height and waist to hip ratio of the subjects were measured, and the body mass index (BMI, kg/m^2) was calculated. Blood pressure was measured in sitting position at the right upper arm with a random-zero sphygmomanometer.

Dexamethasone suppression tests Two dexamethasone suppression tests (DST) were performed. As described previously [23], venous blood was obtained between 8 and 9 am after an overnight fast for serum cortisol and insulin measurements. Participants were instructed to ingest a tablet of 1 mg DEX at 11.00 p.m. The next morning fasting blood for cortisol, insulin, and DEX measurement was drawn by venapuncture at the same time as the previous morning.

In order to get more information about the individual variability of the feedback sensitivity of the HPA-axis all 216 subjects were invited for a second DST with a lower dose DEX (0.25 mg) two and a half years later. Of a total of 155 subjects who agreed to participate in this second test, *TthIII*I genotypes were available (72 men and 83 women).

Hormone Measurements Serum cortisol concentrations were determined using RIA-kits obtained from Diagnostics Products Corporation (Los Angeles, CA). Intra- and interassay variations were below 8.0 % and 9.5 % respectively. The DEX concentration was measured in a radioimmunoassay using antiserum obtained from IgG Corporation (Nashville, TN) to check for compliance and possible abnormalities in the metabolism of DEX. Intra- and interassay variations were below 8.5 % and 14.2 % respectively. Circulating insulin concentrations were determined using commercially available radioimmunoassays (Medgenix Diagnostics, Brussels, Belgium). Intra- and interassay variations were 8.0 % and 13.7 % respectively.

Biochemical Measurements Glucose, total cholesterol, high-density lipoprotein (HDL)-cholesterol and triglycerides were measured using standard laboratory methods. Low-density lipoprotein

(LDL)-cholesterol was calculated using the following formula: LDL-cholesterol = total cholesterol - ((triglycerides/5) + HDL-cholesterol).

Sequencing analysis A *TthIII*I recognition site possibly involved in the *TthIII*I RFLP [21] was identified in Genbank sequence NT_030707. A fragment including this site was PCR amplified from 20 random DNA samples using the primers (forward) 5'-TCCAGGAGTGGGACATAAAGCT-3' and (reverse) 5'-CTTAGAAGCAGAGGTGGAAATGAAG-3' (Biosource Europe S.A., Nivelles, Belgium). The PCR fragments were digested with *TthIII*I enzyme (Promega Corporation, WI, USA) and analyzed on agarose gels. The *TthIII*I site was indeed found to be polymorphic. Subsequently, the sequence of the fragments was analyzed: purified PCR products were sequenced on an ABI Prism 310 Genetic Analyzer, using a BigDye™ Terminator Cycle Sequencing Ready Reaction DNA sequencing kit (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands) according to manufacturer's protocol.

Genetic analysis DNA was extracted from peripheral blood leukocytes using standard techniques. Allelic discrimination was performed to determine genotypes, using TaqMan Universal PCR master mix (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands), primers (see above), probes and a Taqman ABI Prism 7700 Sequence Detection System (Applied Biosystems). The primers used were 5'-TCCAGGAGTGGGACATAAAGCT-3' (forward) and 5'-CTTAGAAGCAGAGGTGGAAATGAAG-3' (reverse). Used probes were 5'-FAM-TGTATTCAGACTCAGTCAAGGCAAGGACC-BHQ1-3' (wild type, Biosource Europe) and 5'-VIC-TGTATTCAGACTCAATCAAGGCAAGGACC-TAMRA-3' (mutant, Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 64.8 °C and optimized concentrations for primers and probes of 100 nmol/L and 100 pmol/μL, respectively. To confirm genotypes, restriction fragment length polymorphism (RFLP) analysis was also carried out, using *TthIII*I restriction enzyme and a digestion of 1 hour at 65 °C, and identical genotypes were found. Genotyping of the N363S and ER22/23EK polymorphisms were performed using RFLP analysis, as previously described [2, 19]. The *Bcl*I polymorphism was determined using allelic discrimination, as also described earlier [17].

Statistical analysis Data was analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, IL). Logarithmic transformations were applied to normalize variables and to minimize the influence of outliers. Differences in means between carriers and noncarriers of the polymorphisms were adjusted for age and sex and tested by ANCOVA using the general linear model procedure. Two age groups were constructed based on the median age to compare genotype frequencies between these age groups and the diffence was calculated using a Chi square test. Correlations between age and cortisol levels and glucocorticoid sensitivity were calculated using Spearman's correlation coefficient analysis.

Results are reported as mean ± SE. *P* values are two-sided throughout, and a $p < 0.05$ was considered to indicate a significant difference.

Results

Identification of TthIIIΙ polymorphism

The previously described *TthIII*Ι restriction fragment length polymorphism was found to be a C/T single nucleotide polymorphism in the promoter of the *GR* gene, 3807 nucleotides upstream of the GR mRNA start (as in GenBank NM_000176). In previous studies the *TthIII*Ι RFLP was detected by Southern blotting of *TthIII*Ι -digested total genomic DNA. PCR-RFLP and sequence analysis showed that the site (3807 bp upstream of the GR mRNA start) was indeed polymorphic (A<u>C</u>T → A<u>T</u>T) .We observed allelic frequencies similar to those reported previously for the *TthIII*Ι polymorphism. Genotyping revealed in the study population of 209 subjects a total of 83 persons (39.7 %) who were homozygous for the C-allele (CC-carriers), 93 (44.5 %) who were heterozygous carriers of the C-allele and the T-allele (CT-carriers) and 33 (15.8 %) were homozygous for the T-allele (TT-carriers). The allelic frequency of the variant T-allele in this group was 38 %. Genotype distributions did not differ from those expected under Hardy-Weinberg equilibrium conditions.

Table 1: Age, anthropometric parameters and blood pressures in noncarriers (CC), heterozygous *TthIII*Ι carriers (CT), and homozygous *TthIII*Ι carriers (TT).

Genotype	TthIIII CC		TthIIII CT		TthIIII TT		
N	83		93		33		
	Mean	SE	Mean	SE	Mean	SE	P*
Age (yrs)	65.8	0.72	67.5	0.55	67.4	1.02	0.13
Weight (kg)	77.0	1.53	73.6	1.16	75.1	2.31	0.22
Height (cm)	170	1.00	168	0.95	168	1.75	0.74
BMI (kg/m2)	26.6	0.40	26.0	0.37	26.5	0.77	0.51
WHR	0.92	0.11	0.92	0.10	0.95	0.02	0.20
SBP (mmHg)	137.8	2.10	139.4	2.05	145.1	3.33	0.25
DBP (mmHg)	75.0	1.13	74.2	1.02	77.7	1.78	0.20

*Test for differences between the three genotypes. All parameters were adjusted for age and sex, with the exception of age. SE, standard error of the mean, BMI, body mass index, WHR, waist to hip ratio, SBP, systolic bloodpressure, DBP, diastolic bloodpressure.

Sexes were equally represented in the group of CC-carriers (47 % men), as well as in the group of CT-carriers (51% men) and TT-carriers (46% men). Age was not significantly different between the three *TthIII*I genotype groups nor did the genotype frequency differ between the younger and older half of the study population. .However, to rule out any influences of differences in age, all parameters were adjusted for age. No significant differences in anthropometric parameters or blood pressure between the groups were present, as shown in table 1. At the second examination after 2.5 years 155 of the initial 209 (74 %) individuals for whom genotypes were available participated: 59 of whom were CC-carriers (51% men), 74 CT-carriers (45% men), and 22 TT-carriers (41% men).

TthIII polymorphism in relation to feedback sensitivity of the HPA-axis

Table 2 shows the concentrations of early morning serum cortisol concentrations before and after administration of 1 mg DEX, the DEX concentration, and the cortisol suppression in reaction to DEX (Δ cortisol) in the three *TthIII*I genotype groups. Three subjects were taking estrogen-containing medication and because of the significant effect on CBG and therefore cortisol concentration they were excluded from the analysis. One male subject had not taken the 1 mg DEX tablet and was excluded as well. There were no significant differences between the CC-carriers and the CT- or TT-carriers in fasting cortisol concentrations, cortisol concentrations after 1 mg DEX, change in cortisol or in the DEX concentrations (table 2). In this study population no correlations between age and cortisol levels ($r= -0.05$, $p= 0.51$) or glucocorticoid sensitivity ($r= -0.07$, $p= 0.32$) were found. Although glucocorticoid sensitivity did not differ between men and women we corrected all analyses for sex (and age).

Also shown in table 2 are the same parameters before and after the administration of 0.25 mg DEX. Again, there were no significant differences in fasting cortisol, cortisol concentrations after 0.25 mg DEX, change in cortisol or in the DEX concentrations.

TthIII polymorphism in relation to insulin, glucose and lipid concentrations

In order to analyze only data from participants with a normal glucose tolerance, subjects who had developed either hyperinsulinemia or diabetes mellitus after the inclusion in the study (fasting insulin values above 25 mU/L or glucose concentrations of more than 7.0 mmol/l [24]) were excluded from this analysis (29 subjects excluded, n = 180, at the first examination). In these 180 subjects together, a significant increase in insulin concentrations in response to the administration of 1 mg DEX was noted (11.5 ± 0.39 mU/L before, and 17.2 ± 0.66 mU/L after DEX administration, respectively $p<0.001$). There were no differences in this increase in serum insulin concentrations between the CC, CT or TT genotype groups ($p=0.78$). Fasting glucose concentrations were not different between the three genotypes. At second examination, insulin

Table 2 : Cortisol and DEX concentrations before and after 1 and 0.25 mg DEX, respectively, in noncarriers (CC), heterozygous *TthIII*I carriers (CT), and homozygous *TthIII*I carriers (TT).

Genotype	TthIIII CC		TthIIII CT		TthIIII TT		
	Mean	SE	Mean	SE	Mean	SE	P*
Number of participants 1 mg DST	81		92		32		
Fasting cortisol (nmol/l)	514.8	16.2	516.9	13.7	504.8	22.2	0.91
Post-DEX cortisol (nmol/l)	27.4	2.7	28.9	3.0	31.3	7.1	0.82
Δ cortisol (nmol/l)	481.7	15.2	485.5	13.6	473.5	24.0	0.88
DEX (nmol/l)	7.2	0.3	7.5	0.4	7.6	0.5	0.97
Number of participants 0.25 mg DST	59		74		22		
Fasting cortisol (nmol/l)	566.9	19.2	542.0	14.4	511.0	24.6	0.15
Post-DEX cortisol (nmol/l)	275.1	19.0	258.8	15.7	232.0	22.2	0.83
Δ cortisol (nmol/l)	291.8	19.1	283.1	18.0	279.1	28.1	0.96
DEX (nmol/l)	2.8	0.2	2.7	0.2	3.7	0.5	0.11

*Test for differences between the three genotype groups. All parameters were log transformed and adjusted for age and sex. At the 1 mg DST, 3 persons were excluded because they had been using estrogens and 1 person was excluded because he had not taken DEX. DST, dexamethasone suppression test, DEX, dexamethasone, SE, standard error.

levels before and after the administration of 0.25 mg DEX in the total group of 114 subjects were not different between the three genotypes.

Serum concentrations of total cholesterol (p=0.67), LDL-cholesterol (p=0.62), HDL-cholesterol (p=0.99) and triglycerides (p=0.71) did not differ between CC-carriers and CT-carriers or TT-carriers at first measurement. Also at the second measurement 2.5 years later no differences in lipid concentrations were found between *TthIII*I genotypes.

***Combination of TthIII*I and ER22/23EK polymorphisms**

The total number of participants of whom both the *TthIII*I and ER22/23EK polymorphisms could be determined was 197. A total of 16 persons were carriers of the ER22/23EK polymorphism. All 16 ER22/23EK carriers were also carrier of at least one T-allele of the *TthIII*I polymorphism. On the other hand, not all *TthIII*I CT- and TT-carriers were carriers of the ER22/23EK-variant. In order to determine the effects of the presence of both *TthIII*I and ER22/23EK polymorphisms in the same individual, we divided our study population into three groups: a group of noncarriers of both polymorphisms (*TthIII*I CC and ER22/23ER; n = 78), a group of carriers of *TthIII*I (CT or TT) but not of the ER22/23EK variant (n = 103) and a group of carriers of both polymorphisms (*TthIII*I CT or TT and ER22/23EK; n = 16).

As shown in table 3, carriers of *TthIII*I CT/TT and ER22/23EK had a higher mean age. To take this into account in the analyses, we corrected for age (as well as for sex). There were no significant differences in anthropometric parameters between these three groups, however weight tended to be lower in carriers of both *TthIII*I T and ER22/23EK variants (table 3).

Figure 1 shows the cortisol levels after the administration of 1 mg DEX, as well as the absolute decrease in cortisol levels. At baseline, there were no differences in fasting cortisol levels between the three genotype groups. However, post-DEX cortisol levels were significantly higher in the group of carriers of the combination of both polymorphisms (*TthIII*I T and ER22/23EK), compared to the two other groups (figure 1A). The decrease in cortisol levels was also significantly lower in carriers of both variants compared to the two other groups (figure 1B). There were no significant differences between the group of noncarriers of both polymorphisms and the group of carriers of the *TthIII*I T variant. DEX concentrations were not different between the three genotype groups (p=0.18).

In the 0.25 mg DST, which was performed 2.5 years later, we found no significant differences between the three groups in fasting (p=0.23) and post-DEX cortisol levels (p=0.69) or in change in cortisol (p=0.38).Fasting insulin levels tended to be lower in the carriers of both polymorphisms (*TthIII*I T and ER22/23EK: 8.7 ± 1.4 mU/L) compared to noncarriers (11.8 ± 0.6) and carriers of only the *TthIII*I T allele (11.6 ± 0.6). However, this did not reach statistical significance (p=0.12). The same pattern in fasting insulin levels was observed after the 1 mg DST, but this was also not significant (p=0.12). There

were no significant differences in changes in fasting insulin or glucose levels between the three groups.

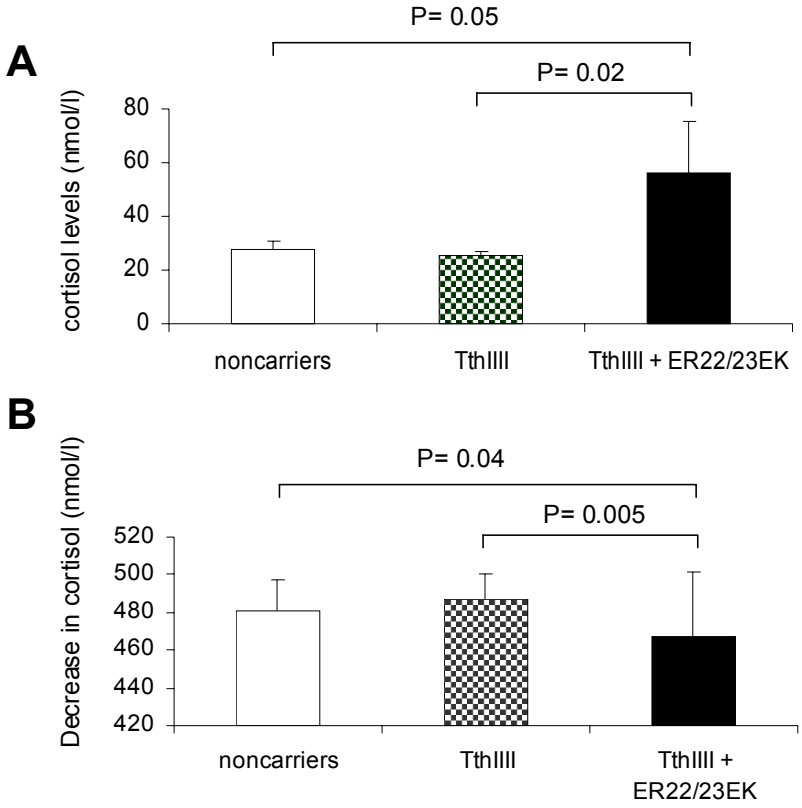

Figure 1: *Glucocorticoid sensitivity in TthIIII-carriers and carriers of both the TthIIII and ER22/23EK variants. (a) Cortisol levels after 1 mg dexamethasone (DEX) were significantly higher in the group of carriers of the combination of both polymorphisms (TthIIII T and ER22/23EK, black bar), compared to the two other groups: noncarriers of both polymorphisms (white bar), and carriers of the TthIIII variant, but not of the ER22/23EK polymorphism (blocked bar). (b) The decrease in cortisol levels was significantly lower in carriers of both variants (black bar) compared to the other groups of noncarriers (white bar) and TthIIII T carriers (blocked bar).*

At the second measurement, before the 0.25 mg DST, we found significantly lower fasting insulin concentrations (p=0.001) in carriers of both polymorphisms (*TthIII*I T+ER22/23EK: 10.0 ± 1.4 mU/L) compared to the groups of noncarriers of both variants (14.8 ± 0.7) and *TthIII*I T carriers (15.2 ± 0.6). No statistically significant differences were found in fasting insulin levels

Table 3: Age, anthropometric parameters and blood pressures in three genotype groups: 1. noncarriers of the *TthIII*I polymorphism and ER22/23EK polymorphism, 2. carriers (heterozygous and homozygous) of the *TthIII*I T-allele, but noncarriers of the ER22/23EK polymorphism, 3. carriers (heterozygous and homozygous) of both the *TthIII*I polymorphism and the ER22/23EK polymorphism (only heterozygous were found).

Genotype	Noncarriers		*TthIII*I **T** carriers		*TthIII*I **T** + ER22/23EK		
N	83		103		16		
	Mean	SE	Mean	SE	Mean	SE	P
Age (yrs)	65.8	0.72	67.0	0.52	70.1	1.58	0.03
Weight (kg)	77.0	1.53	74.8	1.16	69.3	2.81	0.08
Height (cm)	170	1.00	168.7	0.96	166.7	1.82	0.49
BMI (kg/m2)	26.6	0.40	26.3	0.38	25.2	0.95	0.38
WHR	0.92	0.11	0.93	0.09	0.94	0.03	0.95
SBP (mmHg)	137.8	2.10	139.9	1.97	140.7	5.20	0.94
DBP (mmHg)	75.0	1.13	74.5	0.95	78.1	2.96	0.31

Test for differences between the three genotypes. All parameters were adjusted for age and sex, with the exception of age. SE, Standard Error of the mean, BMI, body mass index, WHR, waist to hip ratio, SBP, systolic bloodpressure, DBP, diastolic bloodpressure.

after the administration of 0.25 mg DEX (*TthIIII* T+ER22/23EK: 10.0 ± 1.3, *TthIIII* T: 14.1 ± 0.7, noncarriers: 14.0 ± 0.8, p = 0.34), or change in insulin levels (p = 0.38). Fasting glucose levels tended to be lower in carriers of both polymorphisms (*TthIIII* T+ER22/23EK: 5.3 ± 0.2, *TthIIII* T: 5.5 ± 0.1, noncarriers: 5.7 ± 0.1, however this was not statistically significant (p = 0.14).

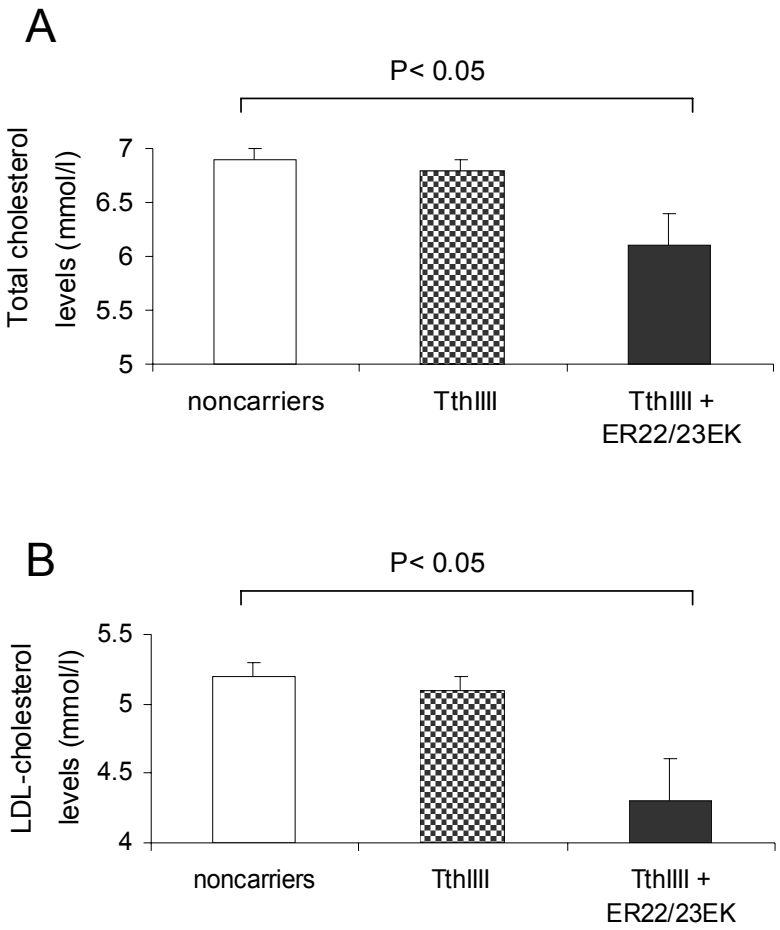

Figure 2: *Cholesterol levels in TthIIII-carriers and carriers of both the TthIIII and ER22/23EK variants. (a) Total cholesterol levels were significantly lower in the group of carriers of the combination of both polymorphisms (TthIIII T and ER22/23EK, black bar), compared to the two other groups: noncarriers of both polymorphisms (white bar), and carriers of the TthIIII variant, but not of the ER22/23EK polymorphism (blocked bar). (b) Low density lipoprotein (LDL)-cholesterol levels were also significantly lower in carriers of both variants (black bar) compared to the other groups of noncarriers (white bar) and TthIIII T carriers (blocked bar).*

Figure 2 shows the cholesterol concentrations in the three genotype groups. Total cholesterol levels were significantly lower in the group of ER22/23EK + *TthIII*I T carriers, compared to noncarriers of both variants and carriers of only the *TthIII*I T variant (figure 2A). LDL-cholesterol levels were also significantly lower in carriers of both polymorphisms (figure 2B). No differences in HDL-cholesterol levels (p=0.96) and triglyceride concentrations (p=0.57) between the three groups were observed.

Two and a half years later, at the second measurement, again total cholesterol levels were lower in carriers of both *TthIII*I T and ER22/23EK (5.6 ± 0.4 mmol/L) compared to noncarriers of the variants (6.5 ± 0.2 mmol/L) and carriers of *TthIII*I T only (6.7 ± 0.2 mmol/L, p<0.05, data not included in figure). Also, LDL-cholesterol levels showed again the same pattern (noncarriers of both variants 4.8 ± 0.2 mmol/L, *TthIII*I T carriers: 4.9 ± 0.2 mmol/L and carriers of both *TthIII*I T and ER22/23EK: 3.9 ± 0.4 mmol/L, p<0.05). Frequency of the *TthIII*I T+ER22/23EK genotype tended to be higher in the older half of our population (13.3% versus 5.1% in the younger half), however this did not reach statistical significance (p= 0.13).

***TthIII*I polymorphism and its relationship to N363S polymorphism**

In order to investigate the effects of carrying both the N363S polymorphism and *TthIII*I polymorphism, we divided our study population into four groups: a group of noncarriers of both polymorphisms (n = 79), a second group of carriers of *TthIII*I CT/TT without N363S (n = 113), a third group of carriers of N363S and *TthIII*I CC (n = 3) and a fourth group of carriers of N363S and *TthIII*I CT/TT (n = 8). Since the numbers of the last two groups were too low we could not perform a reliable statistical analysis on anthropometric parameters, pre- and post-DEX cortisol levels, insulin levels, glucose levels and lipid concentrations.

***TthIII*I polymorphism and its relationship to *Bcl*I polymorphism**

To study a possible interaction between the *TthIII*I and the *Bcl*I polymorphisms, we divided our study population into four groups: a group of noncarriers of both polymorphisms (*TthIII*I CC + *Bcl*I CC; n = 39), a group of carriers of *TthIII*I CT/TT and *Bcl*I CC (n = 39), a group of carriers of *TthIII*I CC and *Bcl*I CG/GG (n = 38) and a group of carriers of *TthIII*I CT/TT and *Bcl*I CG/GG (n = 78). We did not find any significant differences between these four groups in anthropometric parameters, pre- and post-DEX cortisol levels, insulin levels, glucose levels and lipid concentrations.

Possible combinations of GR gene polymorphisms on the same allele

To calculate the possibilities of these four polymorphisms occurring together on the same allele, we used a statistical method for haplotype reconstruction: the Phase Reconstruction Method [25]. Figure 3 shows the alleles that were found: 1.wild type (no polymorphisms), 2. *TthIII*I T without any of the other three polymorphisms we studied in this population, 3. *TthIII*I T with

ER22/23EK, 4. *TthIII*I T with *Bcl*I G, 5. N363S without other polymorphisms, and 6. *Bcl*I G without other polymorphisms.

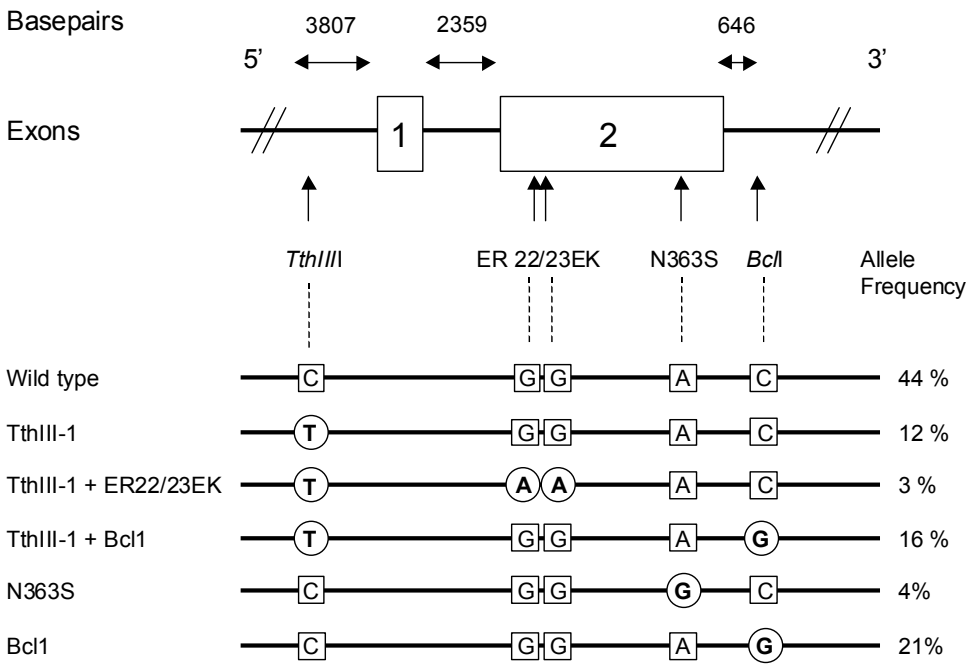

Figure 3: *Overview of variant alleles of the glucocorticoid receptor gene. Schematic overview of the alleles of the GR gene present in the Caucasian population and their calculated frequencies, showing the polymorphisms we investigated in the present study. White squares indicate the wild type nucleotides, and the black squares indicate the variant nucleotides. Bp, basepairs.*

Discussion

In the present study we identified the *TthIII*I polymorphism as a C toT mutation 3807 bp upstream of the mRNA start site of the *GR* gene. We did not observe an altered response in cortisol levels to the administration of DEX in carriers of the *TthIII*I polymorphism, which suggests that this variant is not related to a change in sensitivity to GCs. In line with this, we

did not observe any differences in anthropometric parameters, glucose and insulin concentrations or in lipid levels.

However, we found that carriers of the ER22/23EK polymorphism, who previously had been shown to have a decreased GC sensitivity, and lower insulin, cholesterol [19] and CRP levels [20], always carried the *TthIII*I T variant. Using a statistical method for haplotype reconstruction, we calculated that the ER22/23EK polymorphism is located on the same allele as the *TthIII*I T polymorphism. Carriers of both *TthIII*I T and ER22/23EK showed a reduced cortisol decrease in response to 1 mg DEX, as well as higher post-DEX cortisol levels compared to *TthIII*I T only carriers and noncarriers of both polymorphisms. This suggests that carriers of the combination of both variants are relatively resistant to the effects of GCs. In accordance, we found lower fasting insulin levels, as well as lower total and LDL-cholesterol levels in carriers of both *TthIII*I T and ER22/23EK variants. The carriers of both *TthIII*I T and ER22/23EK polymorphisms also had a higher mean age, which might be a result of their beneficial cardiovascular profile. This is supported by a recent study, in which we found that the ER22/23EK polymorphism is associated with longevity in elderly men [20]. Thus, the *TthIII*I T variant seems not to be functional by itself, but only in combination with the ER22/23EK polymorphism.

It is unclear whether the presence of the *TthIII*I T-allele contributes to the effects of ER22/23EK or whether it is a coincidence that the ER22/23EK mutation has arisen on a *TthIII*I T allele. The observation that there are no carriers of a *TthIII*I C allele with an ER22/23EK mutation on the same allele, suggests that the mutations in codons 22 and 23 have arisen de novo on the highly frequently occuring *TthIII*I T allele.

Rosmond et al showed previously an elevated basal cortisol secretion in *TthIII*I CT- and TT-carriers compared to CC-carriers, without differences in anthropometry, insulin levels, glucose levels, cholesterol levels and systolic and diastolic blood pressure [22]. We suggest that this previously observed association of *TthIII*I CT/TT with elevated basal cortisol secretion, might be explained by a relatively large number of ER22/23EK-carriers within the group of *TthIII*I T carriers of that study population. The frequency of *TthIII*I in the study population of Rosmond et al (284 middle-aged men) was: CC 49.6%, CT 41.4% and TT 9.0%, compared to CC 39.7%, CT 44.5% and TT 15.8% in our study population. However, the number of ER22/23EK-carriers in the population of Rosmond et al is unknown. The different findings on basal cortisol levels in our study could also be explained by the point of time at which the measurements were performed. In the previous study, *TthIII*I T-carriers were found to have elevated diurnal cortisol levels, which where most pronounced at night [22]. In our study we only measured early morning cortisol levels. Alterations in glucocorticoid sensitivity due to a receptor variation could result in a compensatory response of the HPA-axis to adjust the total cortisol production rate. However, we did not find any significant differences in basal cortisol levels. This could be due to the large interindividual variation in morning cortisol levels. However, the phenotypic changes we observed are more likely to be due to tissue specific

changes in the the receptor sensitivity. In future research it would be interesting to investigate whether the *TthIII*I T genotype with, as well as without the ER22/23EK variant is related to midnight cortisol levels, and whether any effects of the polymorphisms can be observed in a stimulation test with ACTH.

We did not find any interaction between *TthIII*I T and *Bcl*I G polymorphisms. Since the frequency of the combined carriage of *TthIII*I T and N363S polymorphism.s was low we cannot draw any conclusions with respect to glucocorticoid sensitivity or phenotpic changes. However, we found that the *TthIII*I T and N363S variants did not occur on the same allele in our population, which suggests that the original N363S mutation arose on a *TthIII*I C allele.

Interestingly, the three previously described polymorphisms, ER22/23EK, N363S, and *Bcl*I, which all are associated with altered GC sensitivity, never occurred on the same allele. It is most likely, that the original de novo mutations all arose separately either on a wild type allele or, in the case of the ER22/23EK polymorphism, on a *TthIII*I variant allele. However, *in vitro* experiments are necessary to determine what the effects are of the presence of the exonic polymorphisms at the same allele, and whether these effects might be deleterious and therefore not compatible with life. Further research is necessary to clarify the mechanisms at the molecular level through which the known *GR* gene polymorphisms exert their effects.

We conclude that the *TthIII*I polymorphism itself is not associated with a change in sensitivity to GCs. However, the ER22/23EK polymorphism is invariably linked to the *TthIII*I T variant, and this *TthIII*I T+ ER22/23EK haplotype is associated with a relative resistance to GCs, as well as lower total and LDL-cholesterol levels, and lower fasting insulin levels in our elderly population. The three previously described functional *GR* gene polymorphisms (ER22/23EK, N363S and *Bcl*I) appear not to occur on the same allele, which facilitates the interpretation of association studies on these variants.

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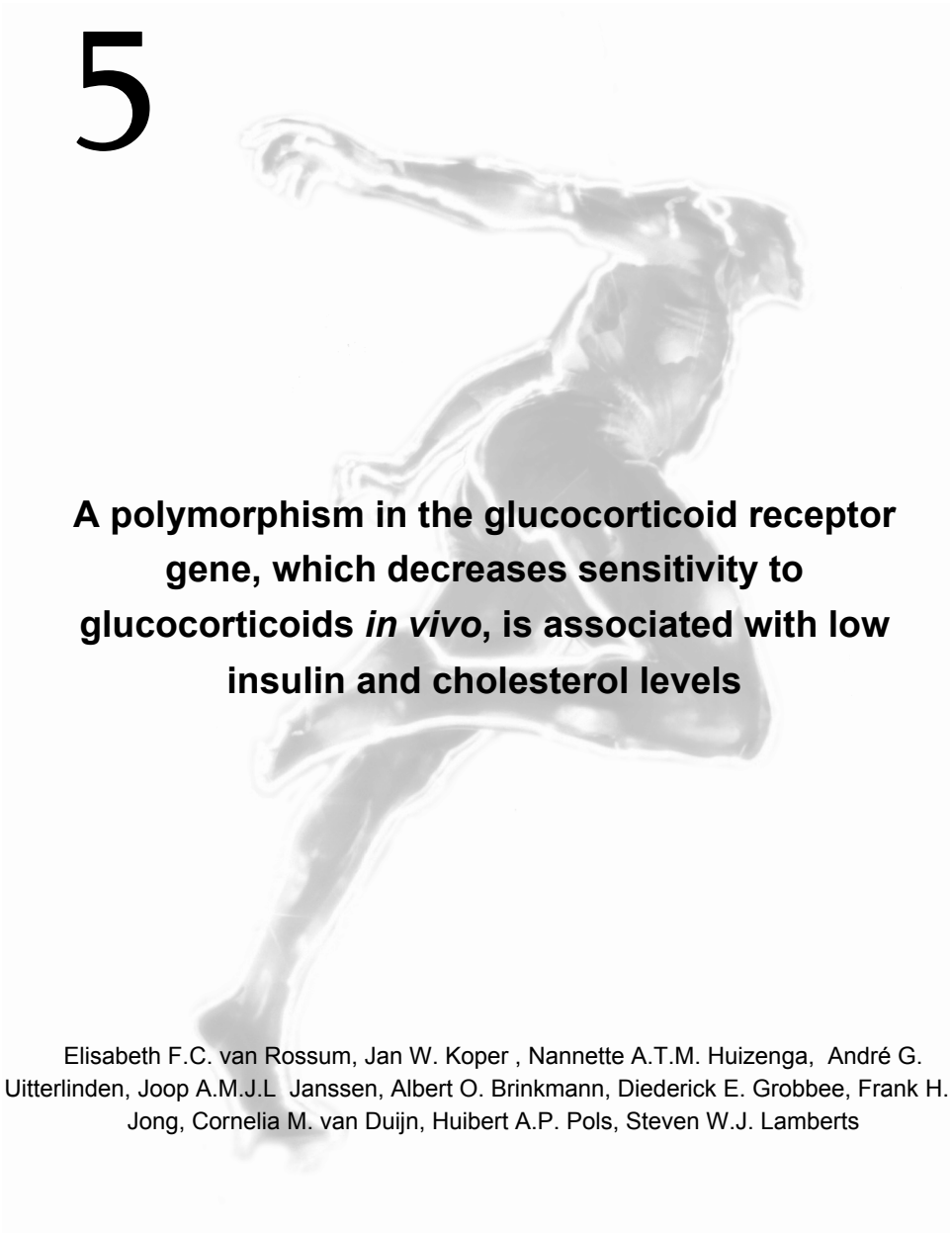

A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids *in vivo*, is associated with low insulin and cholesterol levels

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Abstract

We investigated whether a polymorphism in codons 22 and 23 of the glucocorticoid receptor gene (GAGAGG(GluArg) to GAAAAG(GluLys)) is associated with altered glucocorticoid sensitivity, anthropometric parameters, cardiovascular risk factors and sex steroid hormones. In a subgroup of 202 healthy elderly subjects of the Rotterdam Study, we identified 18 heterozygotes (8.9 %) for the 22/23EK-allele (ER22/23EK-carriers). In the highest age group the number of ER22/23EK-carriers was higher (67-82 years: 12.9%) than in the youngest age group (53-67 years: 4.9%; $p<0.05$). Two dexamethasone suppression tests with 1 and 0.25 mg dexamethasone (DEX) were performed and the serum cortisol and insulin concentrations were compared between ER22/23EK-carriers and non-carriers. After administration of 1 mg DEX the ER22/23EK group had higher serum cortisol concentrations (54.8 ± 18.3 vs. 26.4 ± 1.4 nmol/L; $p<0.0001$), as well as a smaller decrease in cortisol (467.0 ± 31.7 vs. 484.5 ± 10.3 nmol/L; $p<0.0001$). ER22/23EK-carriers had lower fasting insulin concentrations ($p<0.001$), total ($p<0.02$) and LDL-cholesterol concentrations, ($p<0.01$). No other differences between the genotypes were found in anthropometric parameters, blood pressure and circulating IGF-BP1, HDL-cholesterol, triglycerides or sex hormones. Our data suggest that carriers of the 22/23EK allele are relatively more resistant to the effects of glucocorticoids with respect to the sensitivity of the adrenal feedback mechanism than non-carriers, resulting in a better metabolic health profile.

Introduction

Glucocorticoids (GC) are important regulators in almost every tissue in the human body and their effects are mediated by the glucocorticoid receptor (1). A complete inability of GCs to exert their effects on target tissues is probably not compatible with life. However, several patients have been described with partial forms of GC resistance. They show a wide spectrum of clinical symptoms, such as hypertension, hypokalemic alkalosis (2), fatigue and hyperandrogenism (3). Vingerhoeds et al. reported a father and a son with GC resistance (2) and from then on 20 additional patients and family members with this syndrome have been described (4).

Besides these symptomatic patients with relative glucocorticoid resistance, within the normal population a considerable variability in the feedback sensitivity of the hypothalamo-pituitary-adrenal (HPA-)axis was also demonstrated (5). The molecular mechanisms underlying this variation in GC- sensitivity are still largely unknown. In the symptomatic patients with familial forms of glucocorticoid resistance missense mutations in the ligand binding domain of the glucocorticoid receptor (GR) gene causing decreased ligand binding affinity have been described (6, 7), as well as a deletion of four base pairs at the boundary of exon 6 and intron 6, causing loss of a splice site and a 50% reduction of receptor number per cell resulting also in GC-resistance (8). Within the normal population, several polymorphisms in the GR gene have been reported (9). One of these polymorphisms consists of a point mutation in codon 363 in exon 2 of the GR gene, resulting in an asparagine to serine amino acid change, and was shown to be associated with an increased sensitivity to GCs in response to dexamethasone (DEX) (10). Another polymorphism consists of two linked point mutations separated by one base pair in codon 22 and 23 in exon 2 of the GR gene. The mutations are located at cDNA positions 198 and 200, respectively. The first mutation is silent, changing codon 22 from GAG to GAA, both coding for glutamic acid (E). The second mutation changes codon 23 from AGG to AAG, resulting in an amino acid change from arginine (R) to lysine (K) (9). These mutations did not seem to alter the activity of the GR in 'in vitro' experiments (11). However, the clinical relevance of this polymorphism has not been studied. Within the context of an ongoing population based cohort study of diseases in the elderly (The Rotterdam Study) we investigated whether in vivo there were any differences between ER22/23EK-carriers and non-carriers in the sensitivity of the HPA- axis to the overnight administration of 1 mg or 0.25 mg dexamethasone, as well as in some anthropometric parameters, cardiovascular risk factors and in sex steroid hormone levels.

Subjects and Methods

Subjects A total of 202 persons participated in the study. Their age varied between 53 and 82 years (98 men and 104 women with mean ages of 67.7 ± 0.6 and 65.9 ± 0.6 years, respectively). They were living in a suburb of Rotterdam, The Netherlands. These subjects were participants in the Rotterdam Study, a population-based cohort study (7983 subjects) of the determinants of chronic disabling diseases in the elderly and were at random selected. Subjects with acute, psychiatric or endocrine diseases, including diabetes mellitus treated with medication, were not invited. Compared to all participants of the Rotterdam study, there were no differences in age and gender distribution and cardiovascular risk factors. The subjects gave their written consent to participate in the study which received the approval of the Medical Ethics Committee of the Erasmus University Medical School. In order to get more information about the individual variability of the feedback sensitivity of the HPA-axis all 202 subjects were invited for a second DST with a lower dose DEX (0.25 mg) two and a half years later. 149 subjects agreed to participate in this second test (72 men and 77 women).

Anthropometric Measurements Body weight, height and waist to hip ratio of the subjects were measured, and the body mass index (BMI, kg/m^2) was calculated. Blood pressure was measured in sitting position at the right upper arm with a random-zero sphygmomanometer.

Dexamethasone suppression tests The two dexamethasone suppression tests (DST) were performed as described previously (5). In brief, venous blood was obtained between 8 and 9 am after an overnight fast for serum cortisol and insulin measurements. Participants were instructed to ingest a tablet of 1 mg (and 0.25 mg for the second DST) DEX at 11.00 pm. The next morning fasting blood was drawn by venapuncture at the same time as the previous morning. To check for compliance and possible abnormalities in the metabolism of DEX, the DEX concentration was also measured in a radioimmunoassay using antiserum obtained from IgG Corporation (Nashville,TN). Intra- and interassay variations were below 8.5 % and 14.2 % respectively.

Hormonal Measurements Serum cortisol concentrations were determined using RIA-kits obtained from Diagnostics Products Corporation (Los Angeles, CA). Intra- and interassay variations were below 8.0 % and 9.5 % respectively. Circulating insulin and cortisol binding globulin (CBG) concentrations were determined using commercially available radioimmunoassays (Medgenix Diagnostics, Brussels, Belgium). Intra- and interassay variations were 8.0 % and 13.7 % respectively. Estradiol, androstenedione and dehydroepiandrosterone-sulfate (DHEAS) concentrations were determined using RIA-kits obtained from Diagnostics Products Corporation. Intra- and interassay variations; estradiol: 7.0 % and 8.1 %, androstenedione: 8.3 % and 9.2 %, DHEAS: 5.3% and 7.0%. Sex hormone binding globulin (SHBG) was assayed with a commercially available immunoradiometric assay (Diagnostics Products Corporation; intra- and interassay variations were 3.6 % and 6.9 % respectively).

Testosterone was measured with a non-commercial radioimmunoassay (intra- and interassay variations 3.6 % and 6.9 %). Commercially available immunoradiometric assays were used for the measurement of insulin-like growthfactor-binding protein-1 (IGF-BP1, Diagnostic System Laboratories Inc.; intra- and interassay variations 4.0 % and 6.0 %). Insulin resistance (IR) and beta cell function (B) were estimated using the Homeostasis Model Assessment (HOMA), as previously described (12, 13).

Biochemical Measurements Glucose, total cholesterol, high density lipoprotein (HDL)-cholesterol and triglycerides were measured using standard laboratory methods. Low density lipoprotein (LDL)-cholesterol was calculated using the following formula: LDL-cholesterol = total cholesterol - ((triglycerides/5) + HDL-cholesterol).

Genetic analysis Restriction fragment length polymorphism analysis was carried out to determine GR genotypes. DNA was extracted from peripheral blood leukocytes using standard techniques. PCR amplification of the GR gene was carried out employing primer sequences and amplification conditions as described previously (9). The PCR-products were digested with 1 U Mnl I (New England Biolabs, Inc) at 37 ºC for 1 hour. Mnl I cleaves at 5'-CCTC(N)7-'3 and at 3'-GGAG(N)6-'5. Fragments were visualised with ethidium bromide on a 3% agarose -gel (MP-Boehringer, Mannheim). We re-analysed the 18 heterozygous and 10 wild type samples and found identical genotypes.

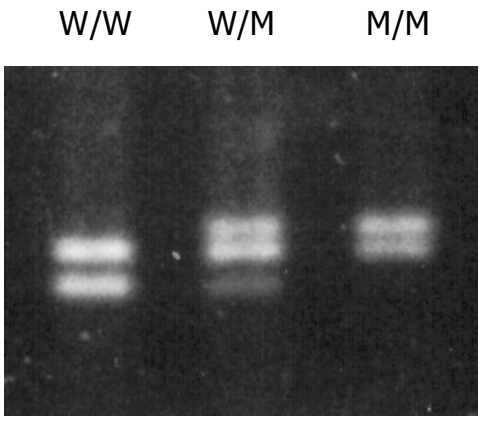

Figure 1: *Representative electrophoretic pattern of the GR22/23 genotype analysed by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) from three subjects with the wildtype ER22/23ER (WW, lane 1), heterozygous ER22/23EK (WM, lane 2) and homozygous EK22/23EK (MM, lane 3) genotypes.*

The ER22/23ER yielded a 163 and a 142 bp fragment, the ER22/23EK appeared as three fragments of 177, 163 and 142 bp, while the EK22/23EK consisted of a 177 and 163 bp fragment. (The homozygously affected individual was discovered in a related population study in young children. Preliminary investigations did not reveal specific anthropometric phenotypic changes in this individual)

Statistical analysis Data were analyzed using SPSS for Windows, release 9.0 (SPSS, Chicago, IL). Logarithmic transformations were applied to normalize variables and to minimize the influence of outliers. Differences between the ER22/23EK-carriers and the non-carriers were adjusted for age and sex and tested by ANCOVA using the general linear model procedure. A paired samples t-test was used to compare changes in insulin concentrations before and after the administration of DEX in all subjects. Results are reported as mean ± SE.

Pearson's correlation coefficients were used to calculate correlations between cortisol, insulin and cholesterol after correction for age, sex and, if necessary, for BMI. The two age-groups were chosen based on the median age (67.02). Comparison of the frequencies of the genotypes between different age-groups was carried out using a Chi-square test. *P* values are two-sided throughout, and a $p < 0.05$ was considered to indicate a significant difference.

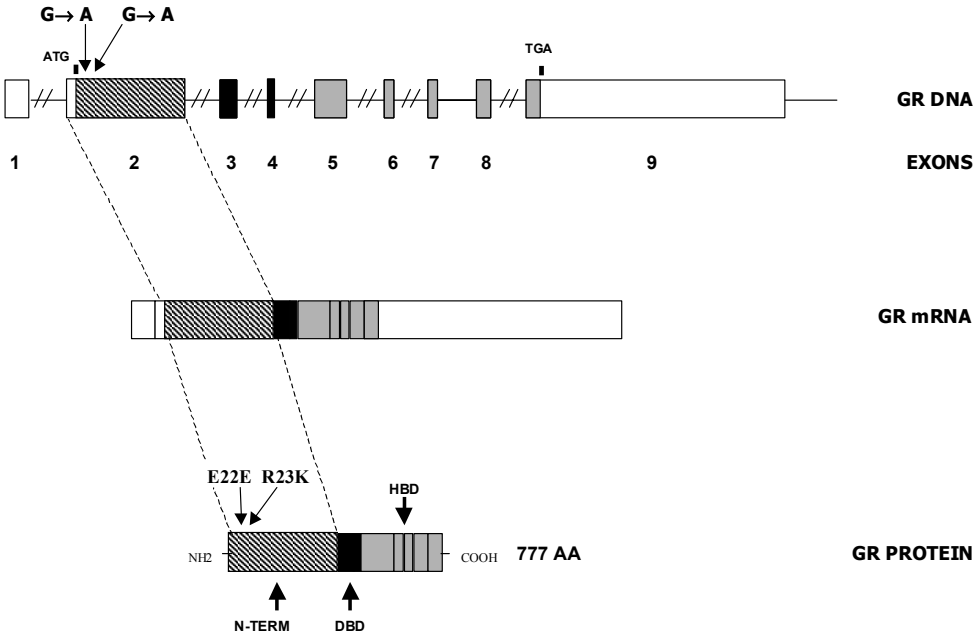

Figure 2: *Structure of the human glucocorticoid receptor gene, mRNA and protein and its functional domains. The position of an arginine to lysine change at codon 23 as a result of the G to A point mutation and the silent point mutation of a G to A at codon 22 are indicated. N-TERM, NH_2- terminal domain, DBD, DNA binding domain, HBD, hormone binding domain.*

Results

Restriction fragment length polymorphism analysis revealed in the study population of 202 subjects a total of 18 persons (8.9 %) who were heterozygous for the polymorphism in codon 22/23 (see also Figures 1 and 2). No individuals homozygous for this polymorphism were found in this group. The allele frequency of the variant allele in this group was 4.5 %. Genotype distributions did not differ from those expected under Hardy-Weinberg equilibrium conditions, however we cannot say this with absolute certainty, as we did not find any homozygous ER22/23EK-carriers. Sexes were equally represented in the group of ER22/23EK-carriers (9 men and 9 women), as well as in the group of non-carriers (89 men and 95 women). The ER22/23EK-carriers were 2.7 years older compared to non-carriers, which did not reach statistical significance (Table 1; p= 0.09). However, in the age group between 67 and 82 years (n= 101) the number of ER22/23EK-carriers was higher (12.9 %) than in the age group between 53 and 67 (n=101, 4.9 % ER22/23EK-carriers; p< 0.05). To rule out the influences of differences in age, all parameters were adjusted for age. No significant differences in anthropometric parameters or blood pressure between the groups were present, as shown in Table 1. At the second examination after 2.5 years 149 of the initial 202 individuals participated (74 %), 13 of whom were heterozygous for the codon 22/23 polymorphism. Also in this group of ER22/23EK-carriers the sexes were equally represented (6 men and 7 women). The group of non-carriers now consisted of 66 men and 70 women.

Table 1: Anthropometric parameters and blood pressure in non-carriers (n = 184) and ER22/23EK-carriers (n = 18) at baseline

	Non-carriers			ER22/23EK-carriers			
	Mean	SE	Range	Mean	SE	Range	P*
Age (years)	66.5	0.44	53.0-81.6	69.2	1.68	53.5-82.4	0.09
Height (cm)	170	0.01	146-189	169	0.02	152-175	0.85
Weight (kg)	74.7	1.15	45.8-121.0	71.9	1.97	43-89.9	0.69
BMI (kg/m^2)	26.4	0.28	16.4- 43.1	25.4	0.85	16.4-32.5	0.25
WHR	0.92	0.01	0.66-1.12	0.94	0.02	0.75-1.08	0.62
SBP (mmHg)	138.9	1.42	99-185	140.2	5.03	96-178	0.86
DBP (mmHg)	74.7	0.73	48-97	77.1	2.95	44-97	0.42

*Test for the difference between non-carriers and ER22/23EK-carriers. All parameters were log transformed and, with the exception of age, adjusted for age. SE, Standard Error of the mean, BMI, body mass index, WHR, waist to hip ratio, SBP, systolic bloodpressure, DBP, diastolic bloodpressure.

Feedback sensitivity of the HPA-axis

Table 2 shows the concentrations of early morning serum cortisol concentrations before and after administration of 1 mg DEX, the DEX concentration, and the cortisol suppression in reaction to DEX (Δ cortisol). Three subjects were taking estrogen-containing medication and because of the significant effect on CBG and, therefore, cortisol concentration, they were excluded from the analysis (1 of the ER22/23EK carriers and 2 of the non-carriers)[14]. One male subject had not taken the 1 mg DEX tablet and was excluded as well. There were no differences between the non-carriers and the ER22/23EK-carriers in fasting cortisol concentrations. However, the cortisol concentrations after the 1 mg DST were significantly higher in ER22/23EK-carriers than in non-carriers (54.8 ± 18.3 in ER22/23EK-carriers and 26.4 ± 1.4 nmol/l in non-carriers, p< 0.0001).

Table 2: Cortisol and DEX concentrations before and after 1 and 0.25 mg DEX, respectively, in non-carriers (n= 181 at first examination and n=136 at second examination) and in ER22/23EK-carriers (n= 17 at first examination and n=13 at second examination)

	Non-carriers			**ER22/23EK-carriers**			
	Mean	SE	Range	Mean	SE	Range	P*
1 mg DST							
Fasting cortisol (nmol/l)	514.8	10.7	41-981	521.8	24.9	308-717	0.56
PostDEX cortisol (nmol/l)	26.4	1.4	2-187	54.8	18.3	11-265	<0.0001
Δ cortisol (nmol/l)	484.5	10.3	39-856	467.0	31.7	138-674	<0.0001
DEX (nmol/l)	7.40	0.27	0.8-18.4	6.90	0.85	1.2-12.9	0.26
0.25 mg DST							
Fasting cortisol (nmol/l)	545.3	12.4	47-914	527.2	30.3	325-710	0.78
PostDEX cortisol (nmol/l)	259.5	12.4	14-630	267.5	31.3	83-418	0.57
Δ cortisol (nmol/l)	285.8	13.5	-64-765	259.7	40.7	31-507	0.23
DEX (nmol/l)	2.85	0.13	0.1-8.7	2.88	0.52	0.6-7.1	0.69

*Test for the difference between non-carriers and ER22/23EK-carriers. All parameters were log transformed and adjusted for age and sex. SE, Standard Error of the mean, DEX, dexamethasone, DST, dexamethasone supression test. Three subjects were taking estrogen-containing medication and were excluded from the analysis (1 ER22/23EK carrier and 2 non-carriers). One non-carrier had not taken the 1 mg DEX tablet and was excluded as well for that analysis.

The absolute decrease of serum cortisol concentrations after dexamethasone, as well as the ratio post-DSTcortisol /fasting cortisol, were significantly different (Δ cortisol: 467.0 ± 31.7 in ER22/23EK-carriers and 484.5 ± 10.3 nmol/l in non-carriers, p< 0.0001, ratio postDEX/ preDEX cortisol: 0.111 ± 0.04 in ER22/23EK-carriers and 0.054 ± 0.00 in non-carriers, p=0.003). The significant difference in post-DEX cortisol concentrations was still present after inclusion of the three estrogen-taking subjects. The actual DEX concentrations did not differ in both groups, so the higher post DEX cortisol levels and the smaller change in cortisol after DEX in the ER22/23EK-carriers were not due to differences in the metabolism of DEX. Also, fasting cortisol-binding globulin (CBG) levels were not different in ER22/23EK-carriers and in non-carriers (data not shown).

Also shown in Table 2 are the same parameters before and after the administration of 0.25 mg DEX. Again, there were no significant differences in fasting cortisol. The post-DEX cortisol concentrations and the decrease in cortisol concentrations after the administration of 0.25 mg DEX, as well as the ratio post/pre-DEX cortisol were not significantly different in ER22/23EK-carriers from those in the non-carriers.

Insulin and glucose concentrations

Figure 3a shows the fasting insulin concentrations before and after the administration of 1 and 0.25 mg DEX, respectively. In order to be certain that only the data from subjects with a normal carbohydrate tolerance were analysed, subjects who had developed either hyperinsulinaemia or diabetes mellitus after the inclusion in the study (fasting insulin values above 25 mU/L or glucose concentrations of more than 7.8 mmol/l) were excluded from this analysis (17 non-carriers excluded, n= 167, and 3 ER22/23EK-carriers excluded, n=15 at the first examination). In these 182 subjects together, a significant increase in insulin concentrations in response to the administration of 1 mg DEX was noted (11.5 ± 5.15 mU/L before, and 17.2 ± 8.41 mU/L after DEX administration, respectively p<0.001). There were no differences in this increase in serum insulin concentrations between the control group and the ER22/23EK-carriers (5.7 ± 0.6 versus 5.5 ± 1.3 mU/L).

The fasting insulin concentrations tended to be lower in ER22/23EK-carriers than in non-carriers (p=0.06). The same applied to the fasting serum insulin levels measured after 1 mg DEX (p=0.07). These differences in post DEX insulin concentrations were not due to differences in DEX concentrations between the two groups. Fasting glucose concentrations were not different between the non-carriers and ER22/23EK-carriers (5.71 ± 0.05 versus 5.69 ± 0.16 mmol/L, respectively).

At second examination, 2.5 years later, the fasting insulin levels in ER22/23EK-carriers were significantly lower than in the non-carriers (p<0.001). Insulin levels decreased in the total group of 115 subjects after the administration of 0.25 mg DEX (14.7 ± 0.45 before, and 13.9 ± 0.50 mU/L after DEX administration, respectively, p<0.01). There were no differences in this

decrease in insulin levels between the non-carriers and the ER22/23EK-carriers. After the administration of 0.25 mg DEX insulin concentrations were not significantly different in the ER22/23EK-carriers from those in the non-carriers (p=0.11). Baseline (fasting) glucose levels tended to be lower in the ER22/23EK-carriers than in the non-carriers (5.3 ± 0.20 and 5.6 ± 0.06 mmol/l, respectively; p=0.07). To assess insulin resistance and secretion we used the HOMA-insulin resistance (HOMA-IR) and beta cell function (HOMA-B) index, respectively. As shown in figure 3b, at first examination, ER22/23EK-carriers had significantly lower HOMA-IR scores (2.3 ± 0.33) than non-carriers (3.0 ± 0.11; p<0.05). At the second measurement the same pattern of HOMA-IR was observed (ER22/23EK-carriers: 2.4 ± 0.39 vs. non-carriers 3.8 ± 0.14; p<0.01). HOMA-B scores tended to be lower in ER22/23EK-carriers compared to non-carriers at both observations (carriers: 84.8 ± 11.6, non-carriers:108.2 ± 3.5; p<0.06 and at second examination: carriers: 119.0 ± 17.4 vs. 148.6 ± 4.7; p<0.07).

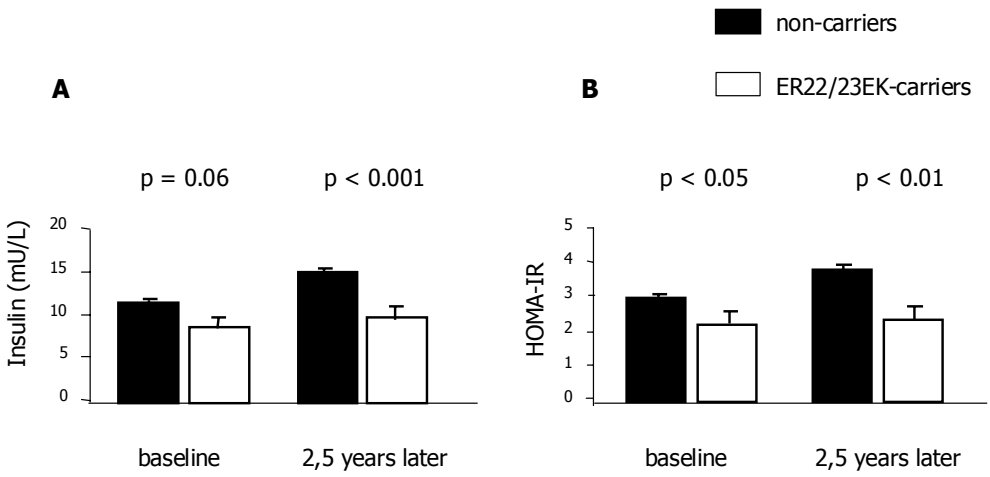

Figure 3: A. *Fasting insulin concentrations in non-carriers (n= 167) and ER22/23EK-carriers (n= 15) before 1 mg DEX at first examination. Insulin concentrations tended to be lower in ER22/23EK-carriers compared to non-carriers (p=0.06). On the right, baseline insulin concentrations in non-carriers (n= 105) and ER22/23EK-carriers (n= 10) at second examination (2,5 years later). Fasting insulin concentrations were significantly lower in ER22/23EK-carriers (p<0.001).* ***B.*** *HOMA-IR scores in non-carriers and ER22/23EK-carriers before 1 mg and 0.25 mg DEX. At both measurements ER22/23EK-carriers were significantly less insulin resistant. All parameters were log transformed and adjusted for age. Subjects with fasting insulin>25mU/l or fasting glucose >7.8 mmol/l were excluded from the calculation; 3 ER22/23EK-carriers and 17 non-carriers were excluded from the first test and 3 ER22/23EK-carriers and 31 non-carriers were excluded from the second test.*

Risk Factors for coronary heart disease and diabetes mellitus

In table 3 serum concentrations of IGF-BP1, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides are shown. There were no differences between non-carriers and ER22/23EK-carriers in IGF-BP1 levels or in HDL-cholesterol and triglyceride concentrations. However, total cholesterol levels were significantly lower in ER22/23EK-carriers than in non-carriers (6.86 ± 0.09 in non-carriers, versus 6.12 ± 0.25 mmol/L in ER22/23EK-carriers, p=0.02), as well as LDL-cholesterol levels (5.11 ± 0.08 mmol/L in non-carriers, versus 4.31 ± 0.25 mmol/L in ER22/23EK-carriers, p< 0.01) At the second examination after 2.5 years serum cholesterol concentrations were again lower (total cholesterol: 6.61 in non-carriers, versus 5.64 mmol/L in ER22/23EK-carriers p=0.01, LDL-cholesterol: 4.87 in non-carriers, versus 3.87 in ER22/23EK-carriers p<0.01, not shown in table).

Table 3: Risk factors for coronary heart disease and Diabetes Mellitus at first examination in non-carriers (n= 184) and ER22/23EK-carriers (n= 18)

	Non-carriers			**ER22/23EK-carriers**			
	Mean	SE	Range	Mean	SE	Range	P
IGF-BP 1 (µg/l)	19.3	1.59	1-154	18.8	2.91	2.0-43.1	0.57
Total chol (mmol/l)	6.86	0.09	2.80-10.32	6.12	0.25	4.31-7.41	0.02
LDL-chol (mmol/l)	5.11	0.08	0.91-8.31	4.31	0.25	2.65-6.09	0.01
HDL-chol (mmol/l)	1.36	0.03	0.72-2.51	1.43	0.14	0.84-2.83	0.63
Triglycerides (mmol/l)	1.91	0.07	0.47-7.42	1.93	0.33	0.76-7.00	0.67

Test for the difference between non-carriers and ER22/23EK-carriers. All parameters were log transformed. and adjusted for age. SE, Standard Error of the mean. IGF-BP1, insulin-like growthfactor-binding protein-1, chol, cholesterol, LDL, low density lipoprotein, HDL, high density lipoprotein

Sex hormones

Table 4 shows the fasting concentrations of sex hormones for men and women separately. No differences between the non-carriers and the ER22/23EK-carriers in the concentrations of estradiol, SHBG, androstenedione, DHEA-S or testosterone were detected. Again, the three subjects who were taking estrogen-containing medication were excluded from the analysis (1 female ER22/23EK carrier and 2 female non-carriers).

Table 4: Hormones at first examination in male (n= 89) and female (n= 93) non-carriers and in male (n= 9) and female (n= 8) ER22/23EK-carriers

	Men					Women				
	non-carriers		ER22/23EK-carriers			non-carriers		ER22/23EK-carriers		
	mean	SE	mean	SE	P	mean	SE	mean	SE	P
Estradiol (pmol/L)	108.0	5.26	88.8	22.8	0.53	82.8	5.39	85.8	17.1	0.79
SHBG (nmol/L)	50.5	2.23	44.0	6.74	0.83	55.4	2.74	62.5	7.99	0.56
Adion (nmol/L)	6.47	0.28	6.26	0.69	0.82	4.45	0.23	4.59	0.88	0.95
DHEA-S (mmol/L)	3.97	0.24	3.80	0.46	0.76	2.38	0.49	2.54	0.16	0.92
Testos (nmol/L)	20.2	0.56	21.9	1.70	0.36	1.37	0.06	2.03	0.71	0.20

Test for the difference between non-carriers and ER22/23EK-carriers. All parameters were log transformed. SE, Standard Error of the mean. SHBG, sex hormone binding globulin, Adion, androtenedione, DHEAS, dehydroepiandrosterone-sulfate,Testos, testosterone. Three subjects were taking estrogen-containing medication and were excluded from the analysis (1 female ER22/23EK carrier and 2 female non-carriers).

Correlations

Degree of cortisol suppression with 1 mg and 0.25 mg DEX positively correlated with fasting insulin ($r = 0.19$; $p<0.02$, $r = 0.22$; $p<0.03$, resp.) and HOMA-IR ($r = 0.21$; $p<0.01$, $r = 0.23$; $p<0.02$, resp.) after adjustment for age and sex. These correlations persisted also after additional correction for BMI (figure 4). No correlations were found between decrease in cortisol and insulin response, total HDL- and LDL-cholesterol, triglycerides or HOMA-B scores. Baseline insulin concentrations correlated positively with triglycerides ($r = 0.31$; $p<0.0001$), while an inverse relation was found with hdl-cholesterol ($r = -0.24$; $p<0.005$).

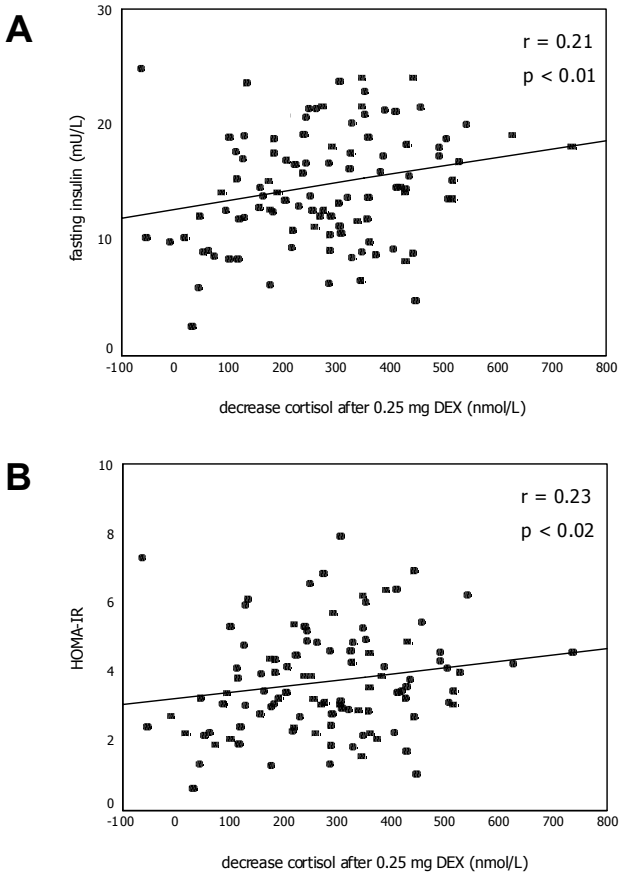

Figure 4: *Correlation between decrease in cortisol after 0.25 mg DEX and (A) fasting insulin and (B) HOMA-IR. Data were adjusted for age, sex and BMI. Subjects who had taken estrogen-containing medication or with fasting insulin>25mU/l or fasting glucose >7.8 mmol/l were excluded from the analysis.*

Discussion

In this population study in the elderly involving 202 individuals we found 18 subjects who were heterozygous for the ER22/23EK polymorphism (8.9 %). Genotype distribution was in Hardy Weinberg equilibrium, as far as we can say without finding any homozygous ER22/23EK-

carriers, suggesting this sample to be at random. The ER22/23EK-carriers had higher serum concentrations of cortisol and a smaller decrease in cortisol concentrations after the administration of 1 mg DEX than non-carriers. We would have expected to find a slight resistance more easily in a 0.25 mg DST than in a 1 mg test. We indeed found the same pattern of smaller decrease in cortisol and higher post-DEX cortisol levels in ER22/23EK-carriers compared to non-carriers in the 0.25 mg DST. However, it was not significant, possibly due to the lower number of subjects who participated in the second test. Furthermore, ER22/23EK-carriers tended to have lower insulin levels before and after a 1 mg DST. These data were partially confirmed two and a half years later with a 0.25 mg DST. Fasting insulin concentrations were again lower in ER22/23EK-carriers than in non-carriers and fasting glucose levels tended to be lower in ER22/23EK-carriers as well. In line with these data, HOMA-IR values were lower in ER22/23EK-carriers, which indicates that they are more sensitive to insulin. Furthermore, cortisol suppression after DEX correlated with fasting insulin and HOMA-IR, which was still significant after adjustment for BMI. These observations suggest that this polymorphism in the GR gene is associated with a slight resistance of the feedback regulation of the HPA-axis.

This relative resistance also results in a lower effect of cortisol on glucose metabolism, resulting in slightly lower glucose concentrations, as well as lower insulin levels. This favourable metabolic profile is supported by the observation that total and LDL-cholesterol concentrations were significantly lower in the ER22/23EK group than in the group of non-carriers. This was confirmed at the second examination. These lower cholesterol concentrations can possibly be partially explained by a reduced cortisol effect. GCs have been demonstrated to influence cholesterol levels by several mechanisms, such as regulation of the uptake of LDL by the liver (15) and influencing human adipose tissue lipoprotein lipase gene expression (16). These outcomes of a relative GC resistance, together with the lower insulin, total and LDL-cholesterol and slightly lower serum glucose concentrations, indicate that ER22/23EK-carriers have a healthier metabolic profile than non-carriers. In this respect, our observation of a significantly higher percentage of ER22/23EK-carriers in the older age group supports the finding of a beneficial metabolic effect of this GR polymorphism.

We found no other differences between the genotypes in anthropometric parameters, blood pressure, and serum levels of IGF-BP1, HDL-cholesterol, triglycerides or sex hormones. Especially this last observation is probably in line with the fact that the ER22/23EK polymorphism in the GR gene is associated with a very mild degree of resistance, because other features previously described in patients with symptomatic glucocorticoid resistance (2) (3) such as hypertension and hypokalemia (related to compensatory ACTH-mediated mineralocorticoid overproduction) or acne and a male pattern of baldness (related to overproduction of adrenal androgens) were not observed. We have previously reported (17) 5 patients who were diagnosed with cortisol resistance. At that time, to get a first impression of

the effect of the ER22/23EK polymorphism we had only genotyped 129 out of the group of 216 subjects who underwent a DEX suppression test. By completing the genotyping of the whole group and finding these subtle indications for cortisol-resistance, we might have to interpret the results found at that time differently. We reported three participants who had abnormal post-DEX cortisol values when considering the limit of 140 nmol/L for normal suppressed cortisol values after 1 mg DST. This cut-off value is clinically used to screen for Cushing's disease, but is probably not useful for determining cortisol insensitivity. Now we are showing that the 18 ER22/23EK-carriers had a significantly smaller mean cortisol response to DEX, indicating a slight resistance to the negative feedback of cortisol. We also reported 2 out of 5 patients, who had a symptomatic cortisol-resistance syndrome and carried the ER22/23EK-polymorphism (17). We cannot say whether this polymorphism was involved in causing the syndrome, but it is possible that it was at least a factor, together with other causative factors.

Previously, we have reported a polymorphism located in codon 363 in exon 2 of the GR, which was associated with a increased sensitivity to cortisol (10). The number of N363S-carriers was not significantly different between the groups of ER22/23EK-carriers and non-carriers (1 (5.5%) N363S-carrier vs 12 (6.5%) N363S-carriers, p=0.32). Moreover, exclusion of the N363S carriers did not alter the results.

In transient transfection assays in COS-1 cells, de Lange et al (11) did not find differences in the way the 23K variant receptor regulated transcription from a number of different promotors. While the polymorphism is not located in the core of the τ_1 transactivation domain (variably defined as amino acid 77 to 262 (18) or 98 to 305 (19)), it is possible that the effects described here are caused by altered interactions with other proteins, that do not play a role in the COS-1 system.

In summary, in this study we observed that subjects who were heterozygous for the 22/23EK allele had significantly higher post DEX cortisol concentrations, a smaller decrease in cortisol concentrations, lower insulin and slightly lower fasting glucose levels, as well as slightly lower post DEX insulin levels than subjects without this GR variant. Furthermore, ER22/23EK-carriers had lower total and LDL-cholesterol levels and were overrepresented in the older age group. These data suggest that ER22/23EK-carriers are relatively more 'cortisol-resistant' than non-carriers, which results in a better metabolic health profile. The exact mechanism through which the ER22/23EK variant of the glucocorticoid receptor establishes these favourable effects remains unclear and is one of the subjects of our ongoing research.

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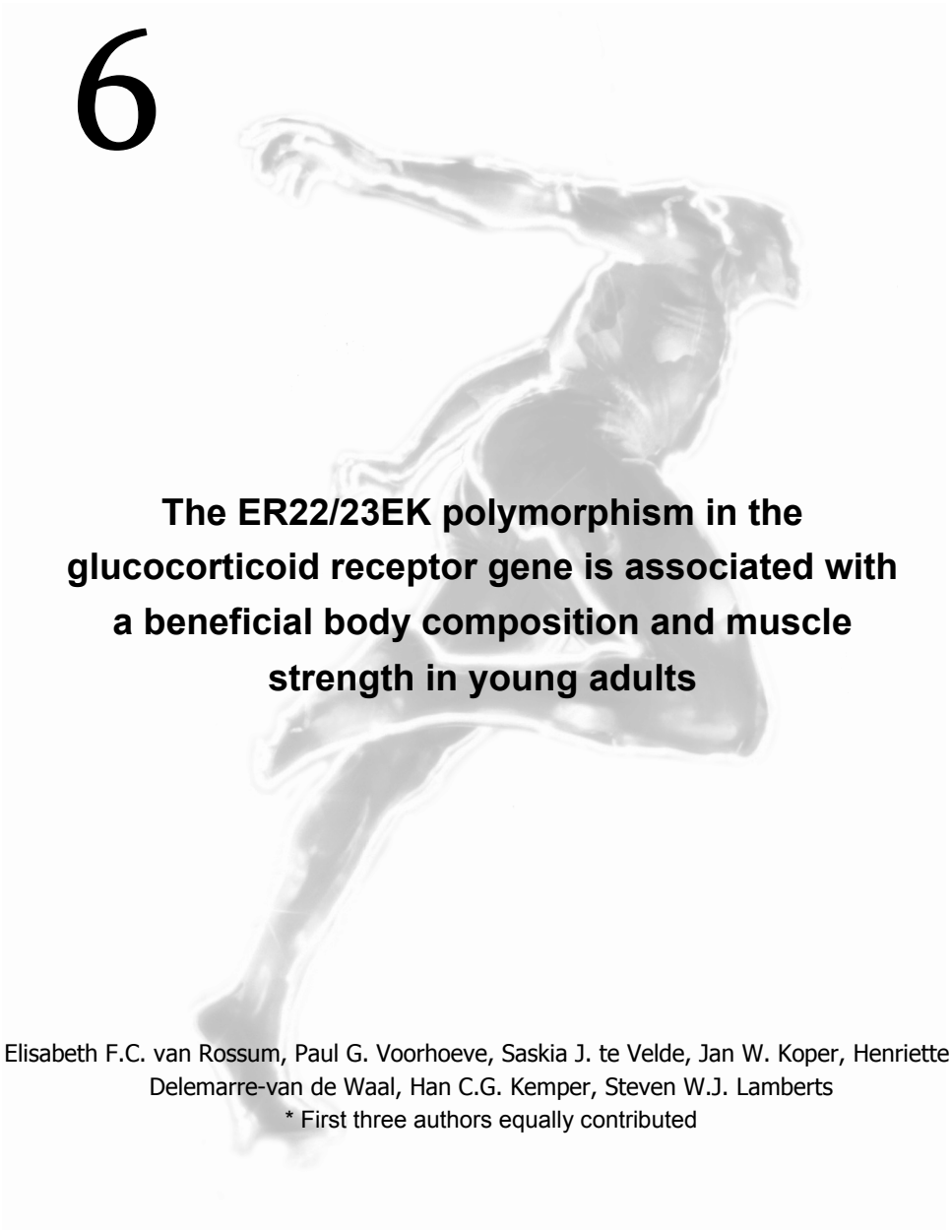

The ER22/23EK polymorphism in the glucocorticoid receptor gene is associated with a beneficial body composition and muscle strength in young adults

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Abstract

Glucocorticoids play an important role in determining body composition. A polymorphism of the glucocorticoid receptor gene (in codons 22 and 23) has previously been found to be associated with relative glucocorticoid resistance, low cholesterol levels, and increased insulin sensitivity. In this study we investigated whether this ER22/23EK polymorphism is associated with differences in body composition and muscle strength. We studied a cohort of 350 subjects who were followed from age 13 until 36 years. We compared noncarriers and carriers of the ER22/23EK variant in anthropometric parameters, body composition, and muscle strength, as measured by arm pull tests and high jump from standing. We identified 27 (8.0 %) heterozygous ER22/23EK-carriers. In males at 36 yrs of age, we found that ER22/23EK-carriers were taller, had more lean body mass, greater thigh circumference, and more muscle strength in arms and legs. We observed no differences in body mass index (BMI) or fat mass. In females, waist and hip circumferences tended to be smaller in ER22/23EK-carriers at the age of 36, but no differences in BMI were found. Thus, the ER22/23EK polymorphism is associated with a sex-specific, beneficial body composition at young adult age, as well as greater muscle strength in males.

Introduction

Glucocorticoids are important regulators in numerous tissues throughout the human body and they also influence body composition. Their effects are mainly mediated by the glucocorticoid receptor (GR), a ligand-activated transcription factor [1]. Thus, changes in the gene coding for this receptor can play an important role in determining glucocorticoid sensitivity [2]. Within the normal population, several polymorphisms in the *GR* gene have been described [3]. One of these polymorphisms - N363S - was shown to be associated with an increased sensitivity to glucocorticoids and a higher body mass index [4], as well as central obesity in males [5]. Lin et al [6] confirmed this finding of higher BMI in N363S carriers and showed an allele-dosage effect of this polymorphism. In contrast, several other studies showed no effect on BMI [7, 8]. A *BclI* polymorphism has previously been shown to be associated with a relative hypersensitivity to glucocorticoids in vivo [9], an increased cortisol response to a standardized lunch, and abdominal obesity in middle-aged subjects [10].

Previously, we identified another polymorphism that consists of two linked point mutations in codons 22 and 23 of the *GR* gene (GAG AGG→ GAA AAG). The first mutation in codon 22 is silent, both GAG and GAA coding for glutamic acid (E). The second mutation changes codon 23 from AGG to AAG, resulting in an amino acid change from arginine (R) to lysine (K) [3]. This polymorphism was associated with a relative resistance to glucocorticoids [11]. We also showed in a population-based study in the elderly that carriers of this ER22/23EK polymorphism had a better insulin sensitivity and lower total and low-density lipoprotein cholesterol levels [11]. In addition, we found the frequency of the 22/23EK allele to be higher in the elder half of the studied population, which suggests a survival advantage. In order to investigate whether the ER22/23EK variant is indeed associated with survival we studied a separate population of 402 elderly Dutch men [12]. After a follow-up of 4 years we found that 19.2 % of the non-carriers had died, while none of the ER22/23EK-carriers (n=21) had died, which was a statistically significant difference. In this same population we also found ER22/23EK-carriers to have lower C-reactive protein (CRP) levels, which in turn were also associated with a better survival. These lower CRP levels in ER22/23EK-carriers possibly reflect a beneficial cardiovascular status [12].

A well-known effect of glucocorticoids is to negatively influence body composition, including redistribution of body fat with deposition of adipose tissue on the abdomen and trunk, and muscle atrophy [13]. It is known that body composition plays an important role in lipid metabolism and insulin sensitivity, and as a consequence influences the risk on cardiovascular disease [14]. At present, it is not known what the effects of this ER22/23EK polymorphism are at a young age or whether there are any effects on body composition.

Therefore, in the present study we investigated a cohort of 350 subjects who were followed from 13 until 36 years, and studied whether there is an association between the

ER22/23EK polymorphism of the *GR* gene and body composition during puberty and at young adult age.

Subjects and Methods

Subjects 350 healthy participants were drawn from the Amsterdam Growth and Health Longitudinal Study (AGAHLS), a population-based observational study with repeated measurements at the age of 13, 14, 15, 16, 27, 29, 32 and 36 years [15]. Subjects of non-Caucasian race were excluded from the analyses (5 males and 5 females, all noncarriers of the ER22/23EK polymorphism). On a total of 337 (158 males) we had complete data on GR genotype and adult anthropometry. Data were not complete at all measurements in puberty: a total of 332 subjects (26 ER22/23EK-carriers) participated at age 13, 290 (22 ER22/23EK-carriers) at age 14, 286 (21 ER22/23EK-carriers) at age 15, and 287 (19 ER22/23EK-carriers) at age 16 years. All subjects gave their written informed consent to participate in the study, which received the approval of the Medical Ethical Committee of the "Vrije Universiteit" of Amsterdam.

Anthropometric Measurements Body weight (kg) was measured to the nearest 0.1 kg using a spring balance scale (Van Vucht, the Netherlands), with subjects dressed only in underwear. Standing height was measured with a stadiometer to the nearest 0.001m. Body mass index (BMI) was calculated as body weight divided by body height squared. To assess fat distribution (abdominal versus gluteo-femoral), we measured waist (at the umbilicus) and hip circumference with a flexible steel tape to the nearest 1 mm and the waist-to-hip ratio (WHR) was calculated. Fat mass (FM) was estimated from four skinfold thickness measurements (biceps, triceps, subscapular and supra iliacal) by the equation of Durnin et al [16, 17, 18]. Lean body mass was measured by dual X-ray absorptiometry (DEXA), with the Hologic QDR-2000 (S/N 2513; Hologic, Inc., Waltham, MA, USA). Calf and thigh circumferences were measured with a steel tape to the nearest 0.1 cm.

Muscle strength was assessed by two physical fitness tests from the MOPER fitness test battery [19 20]. The first was the static arm pull test: the subjects were given two attempts to pull maximally with their arm of preference, the strength of which was measured (in kg) with a dynamometer (Bettendorf, Belgium), fixed to the wall at a horizontal level. The higher score of the two was recorded. The second test was the standing high jump. The subjects had two attempts to jump as high as possible (higher value recorded) from a platform, having been allowed only to bend their knees before jumping. The height they jumped (in cm) was measured by a tape, which was fixed to a belt around the subjects' waist to the platform on the ground.

Physical activity A structured interview based on a physical activity questionnaire (PAQ) was used to investigate the amount of physical activity. The questionnaire comprises questions

about duration, frequency and metabolic (MET) intensity of all physical activities during the last three months preceding the interview. From this information a total weighted activity score (METs/week) was calculated [21 22].

Genetic analysis Restriction fragment length polymorphism analysis was carried out to determine GR genotypes. DNA was extracted from peripheral blood leukocytes by standard techniques. PCR amplification of the *GR* gene was carried out employing primer sequences and amplification conditions as described previously [3]. The PCR-products were digested with 1 U Mnl I (New England Biolabs, Inc) at 37 ºC for 1 hour. Mnl I cleaves at 5'-CCTC(N)7-'3 and at 3'-GGAG(N)6-'5. Fragments were visualized with ethidium bromide on a 3% agarose -gel (Boehringer, Mannheim). We re-analysed all heterozygous and 10 wild type samples and found identical genotypes.

Statistical analysis Data were analyzed by SPSS for Windows, release 10.1 (SPSS, Chicago, IL). Differences in means between the ER22/23EK-carriers and the noncarriers were adjusted for height if appropriate and tested by ANCOVA using the general linear model procedure. High jump scores were corrected for body weight. Results are reported as mean ± SE. *P* values are two-sided throughout, and $p \leq 0.05$ was considered to indicate a significant difference.

Results

Anthropometric parameters at young adult age

In the group of 337 participants we identified 27 (8 %) carriers of the ER22/23EK polymorphism (16 males and 11 females). Table 1 shows anthropometric parameters determined in noncarriers and carriers of the ER22/23EK polymorphism at the last measurement (at the age of 36 years). In males, we found a greater body height in ER22/23EK-carriers (p=0.05), as well as a higher body weight (p=0.03). However, the latter was not significant after adjustment for height (p=0.14). BMI was not different between the genotypes. Total lean mass was significantly higher in ER22/23EK-carriers compared with noncarriers (respectively 66.2 ± 1.5 and 61.4 ± 0.5 kg, p=0.006, after additional correction for height p=0.02). The circumference of the thigh was also greater in ER22/23EK-carriers (ER22/23EK: 60.4 ± 1.1 and noncarriers: 57.4 ± 0.4 cm, p=0.03), while no differences were found in total fat mass or percentage fat.

Table 1 also shows anthropometric parameters in female noncarriers and ER22/23EK-carriers at the age of 36 years. In females, body weight tended to be lower in ER22/23EK-carriers (63.2 ± 1.8 and noncarriers: 68.5 ± 0.8 kg), although this was not statistically significant after adjustment for height (p=0.13). BMI was also not significantly different between the two genotypes (ER22/23EK: 22.0 ± 0.8 and noncarriers: 23.5 ± 0.3, p=0.18). Waist and hip circumferences tended both to be lower in female ER22/23EK-carriers compared

Table 1: Mean and standard error (SE) of anthropometric parameters, muscle strength and MET activity score in non-carriers of both sexes, and in male (n= 16) and female (n=11) ER22/23EK-carriers at the age of 36 years.

	Men (n= 158)					Women (n= 179)				
	non-carriers		ER22/23EK-carriers			non-carriers		ER22/23EK-carriers		
	Mean	SE	Mean	SE	P	Mean	SE	Mean	SE	P
Height (m)	1.83	0.01	1.87	0.02	0.05	1.71	0.01	1.70	0.02	0.48
Weight (kg)	83.5	0.9	89.8	3.1	0.14	68.5	0.8	63.2	1.8	0.13
BMI (kg/m2)	24.8	0.2	25.7	0.8	0.20	23.5	0.3	22.0	0.8	0.18
% total fat	21.5	0.5	21.1	2.0	0.87	32.3	0.6	30.6	2.1	0.36
Total fat mass (kg)	18.1	0.6	19.5	2.4	0.66	22.1	0.6	19.4	1.7	0.26
Total lean mass (kg)	61.4	0.5	66.2	1.5	0.02	42.7	0.4	41.0	1.2	0.38
Thigh circumf (cm)	57.4	0.4	60.4	1.1	0.03	57.2	0.4	55.7	1.0	0.38
Calf circumf (cm)	37.8	0.2	38.7	0.9	0.31	36.1	0.2	35.6	0.6	0.56
Upper arm circumf (cm)	30.5	0.2	31.2	7.3	0.45	27.6	0.2	26.3	0.7	0.15
Waist circumf (cm)	85.0	0.7	87.5	2.1	0.43	73.5	0.7	68.6	1.3	0.07
Hip circumf (cm)	89.0	0.6	91.9	1.8	0.31	89.3	0.7	84.7	2.2	0.09
Arm pull (kg)	70.9	1.1	77.8	3.9	0.06	38.8	0.6	36.2	2.2	0.32
High jump (cm)	51.9	0.6	55.7	1.8	0.04	38.7	0.4	40.1	2.5	0.64
MET-score (mets/week)	4243	267	4678	796	0.60	5316	297	6119	1733	0.49

Test for the difference between non-carriers and ER22/23EK-carriers. All parameters, except the arm pull strength, high jump from standing, and MET score, were corrected for height. High jump scores were adjusted for body weight. SE, Standard Error of the mean. BMI, Body Mass Index, Circumf, circumference, MET, Metabolic Equivalent, a measure of physical activity.

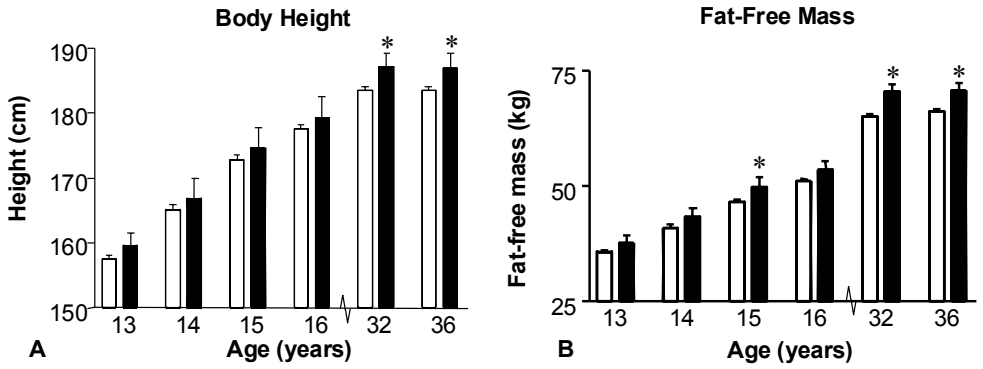

Figure 1: *Height (A) and Fat free mass (B) in male noncarriers (white bars) and carriers of the ER22/23EK polymorphism (black bars) during puberty (age of 13, 14, 15, and 16 years) and adult age (32 and 36 years). ** p≤ 0.05, * p< 0.10*

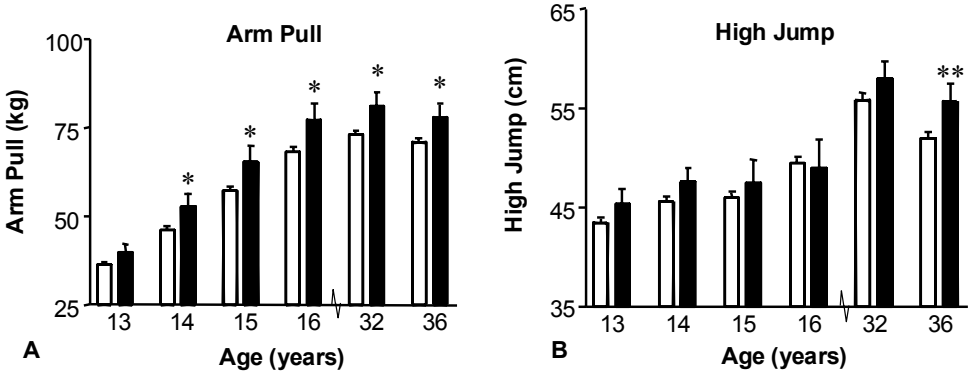

Figure 2: *Arm pull strength (A) and standing high jump (B) in male noncarriers (white bars) and carriers of the ER22/23EK polymorphism (black bars) during puberty (age of 13, 14, 15, and 16 years) and adult age (32 and 36 years). High jump scores were corrected for body weight. ** p≤ 0.05, * p< 0.10*

to noncarriers (p=0.07 and p=0.09, respectively, table 1). No differences were found in height, fat mass and lean body mass, or circumferences of the thigh, calf and upper arm. At the age of 32 years we found similar results (not shown in table). Male ER22/23EK-carriers had a greater body height (p=0.035, figure 1A), higher lean body mass (p=0.02, figure 1B), and higher weight (p=0.006, after adjustment for height (p=0.08)), while total fat mass was not different (p=0.12). In females, we found a tendency towards a smaller waist circumference in female ER22/23EK-carriers (ER22/23EK: 67.5 ± 1.4 and noncarriers: 71.1 ± 0.5, p=0.08). No differences in hip circumference (ER22/23EK: 87.2 ± 2.1 and noncarriers: 90.1 ± 0.7, p=0.27) or in height, weight, BMI, body composition and muscle strength were observed at the age of 32.

Anthropometric parameters in puberty

During puberty we also measured anthropometric variables, body composition and muscle strength in the same subjects. Figure 1A shows the height of male noncarriers and carriers of the ER22/23EK polymorphism during puberty. Although the pattern of greater height in male ER22/23EK-carriers is similar to that at adult age, these differences were not statistically significant. The same applied to the amount of lean mass in males: no significant differences during puberty between genotypes, although a similar pattern as at adult age (higher lean mass in male ER22/23EK-carriers) could be observed (Figure 1B).

At the age of 15, we found tendencies towards higher body weight (p=0.10), BMI (p=0.06), and lean mass (0.09) in ER22/23EK-carriers. On average, male noncarriers grew an additional 5.9 cm after the age of 16, while ER22/23EK-carriers grew 7.8 cm till they reached their final height, however this was not a significant difference. In males, no differences were found in other anthropometric parameters or body composition variables during this period. In females, no differences were observed in the measured parameters at these four measurements during puberty.

Muscle strength at adult age and during puberty

Male ER22/23EK-carriers tended to perform better in the test of arm pull strength (p=0.06 figure 2A), as well as in high jump from standing (adjusted for body weight, p=0.04, figure 2B) at the age of 36 years (see also table 1). Arm pull strength was significantly greater in males at the age of 32 (figure 2A, ER22/23EK: 81.2 ± 3.3 and noncarriers: 73.0 ± 1.1, p=0.02). During puberty, we found the same tendencies towards better arm strength in male ER22/23EK-carriers (figure 2A). Performance on high jump from standing was not significantly different between the genotypes in males at the age of 32 or during puberty (figure 2B). In females, we did not observe any differences in muscle strength of the arm or leg at the age of 36 or 32 years nor during puberty.

Physical activity

At both measurements at young adult age metabolic equivalent (MET)- scores were determined to evaluate physical activity in daily life of the participants. No differences in MET scores between the genotypes were observed in both sexes at age 36 (males: p=0.60, females:p=0.49, table 1) and 32 years (males: ER22/23EK; 2755 ± 279, noncarriers; 3236 ± 255 mets/week, p=0.52, and in females: ER22/23EK; 3687 ± 795, noncarriers; 3547 ± 202 mets/week, p=0.85).

Discussion

In this population-based cohort study in young subjects we identified 8% heterozygous carriers of the ER22/23EK polymorphism. In males at young adult age, we found ER22/23EK-carriers to be on average 4 cm taller than noncarriers and to have significantly more lean body mass, while there were no differences in fat mass. In addition, male ER22/23EK-carriers had greater thigh circumferences, indicating more muscle mass. Functional muscle strength tests showed a better performance of ER22/23EK-carriers in arm strength, with the greatest difference at the age of 32, as well as a better performance in tests concerning strength of the legs. MET-scores did not differ between genotypes, so differences in physical activity did not underlie the greater amount of muscle mass in male ER22/23EK-carriers.

These differences in body composition in males were not yet clearly present during puberty. However, a tendency could be observed towards greater arm strength in male ER22/23EK-carriers during this period, which suggests that the differences already might have existed in puberty. The lack of statistical significance of the other body compositional parameters could possibly be due to the lower numbers of subjects who participated at pubertal age. These incomplete data during puberty might also explain a minor part of the difference in mean height between the age of 16 and age 32 yrs. However, most of this difference in height is explained by natural growth. In The Netherlands boys grow an additional 5.5 cm after the age of 16. Interestingly, male ER22/23EK-carriers grew on average almost 2 cm more than noncarriers after the age of 16. This increased growth suggests that puberty in ER22/23EK-carriers might be extended compared to non-carriers. It is known that glucocorticoids inhibit growth during puberty. Since we found the ER22/23EK polymorphism to be associated with relative glucocorticoid resistance, we would expect less inhibition of growth and thus a greater height. This is in accordance with our finding of a greater height in male carriers of the ER22/23EK polymorphism. Taken together, it remains unclear at what developmental stage exactly these differences between the genotypes that we observed in young adults in height, lean mass and thigh circumference arise. Although the mean heights in this population-based study appear rather tall, these heights are in accordance with the mean

height at these ages in The Netherlands. This suggests that our findings are very well applicable to the Dutch population as a whole.

In young adult females, we found in ER22/23EK-carriers tendencies towards smaller waist and hip circumferences and lower body weight, suggesting a lower amount of subcutaneous fat. These differences could not be detected during puberty. No statistically significant differences were found in measures of body composition or muscle strength between the genotypes.

Longterm exposure to high levels of glucocorticoids are known to negatively influence muscle mass and growth [23, 24]. Thus, the findings of greater height and more muscle mass in male ER22/23EK-carriers could be explained by the observation that ER22/23EK-carriers are relatively resistant to the effects of glucocorticoids, as we recently demonstrated [11]. Another well-known chronic effect of glucocorticoids is redistribution of fat mass to the abdominal region. In line with a glucocorticoid insensitive effect of the ER22/23EK polymorphism we found at the age of 32, as well as at 36 years a tendency towards smaller waist circumference in female ER22/23EK-carriers.

The associations between the ER22/23EK polymorphism and body composition appear to be different between sexes. However, there could be subtle anabolic effects in female ER22/23EK-carriers as well, in line with a relative cortisol resistance and as a result possibly higher androgen levels. When we consider the mean weight difference (more than 5 kg) between female noncarriers and carriers of the ER22/23EK polymorphism, the difference in lean body mass is quite small (less than 2 kg), which indicates that female ER22/23EK-carriers also have relatively more lean body mass. However, the number of female carriers of the polymorphism is relatively small, which might explain that we found no statistically significant differences. Besides more muscle mass, we would also expect less fat mass in subjects with slightly higher androgen levels. In females, we observed tendencies towards smaller waist and hip circumferences, which might reflect a lesser amount of subcutaneous fat mass.

On the other hand, the ER22/23EK polymorphism could have sex-specific effects on body composition. We speculate that differential effects of sex steroid hormones and/ or growth hormone could play a role. It is known, that in rodents the hypothalamic-pituitary-adrenal (HPA) axis is differently regulated in males and in females, both in basal conditions and in response to psychological or physical stress conditions [25]. In this context, androgens inhibit and estrogens enhance the HPA responsiveness to stress [26, 27]. In addition, in a relative glucocorticoid resistant condition, as is the case in carriership of the ER22/23EK variant allele, ACTH production is expected to be slightly higher than in noncarriers due to the lower negative feedback inhibition at the pituitary level. As a consequence, ER22/23EK-carriers might have slightly higher circulating androgen concentrations, which could also, besides a smaller direct (negative) effect of glucocorticoids, contribute to the observed beneficial body composition. The differential effects of sex steroid hormones might explain the gender dimorphism in the

associations we observed between genotype and body composition. However, in the present study we did not measure any serum hormone concentrations.

The exact mechanism of this polymorphism at the molecular level is unknown. The amino acid change in codon 23 (arginine to lysine) might affect the tertiary structure of the receptor. Since the ER22/23EK variant is located near the transactivation domain, this could influence the transactivational and/or transrepressional activity on target genes [28, 29]. Recently, it has been shown that two different GR isoforms (A and B) exist, due to two different methionine (M) codons in the GR mRNA, which both can be used as initation codon (M1 and M27). The GR-B protein has a stronger transactivating effect in transient transfection experiments, but no difference in transrepression [30]. The secondary structure of the GR mRNA might be affected by the ER22/23EK polymorphism, which could result in a different usage of the initiation codons. A change in GR-A/GR-B ratio could then explain the decreased sensitivity to glucocorticoids [29]. Indeed, secondary structure prediction (M-fold) showed different structures for the wildtype and the polymorphic mRNA [29]. A third possibility is that the ER22/23EK polymorphism might lead to differences in binding of proteins, which could affect mRNA stability and thereby influence glucocorticoid sensitivity.

In summary, we found that the ER22/23EK polymorphism of the GR gene is associated with greater body height and more muscle mass and strength in young adult males. In females, we found a tendency towards smaller waist circumference, and to a lesser extent smaller hip circumference. Thus, we conclude that the ER22/23EK polymorphism is associated with a sex-specific, beneficial body composition at young adult age, as well as more muscle strength in males.

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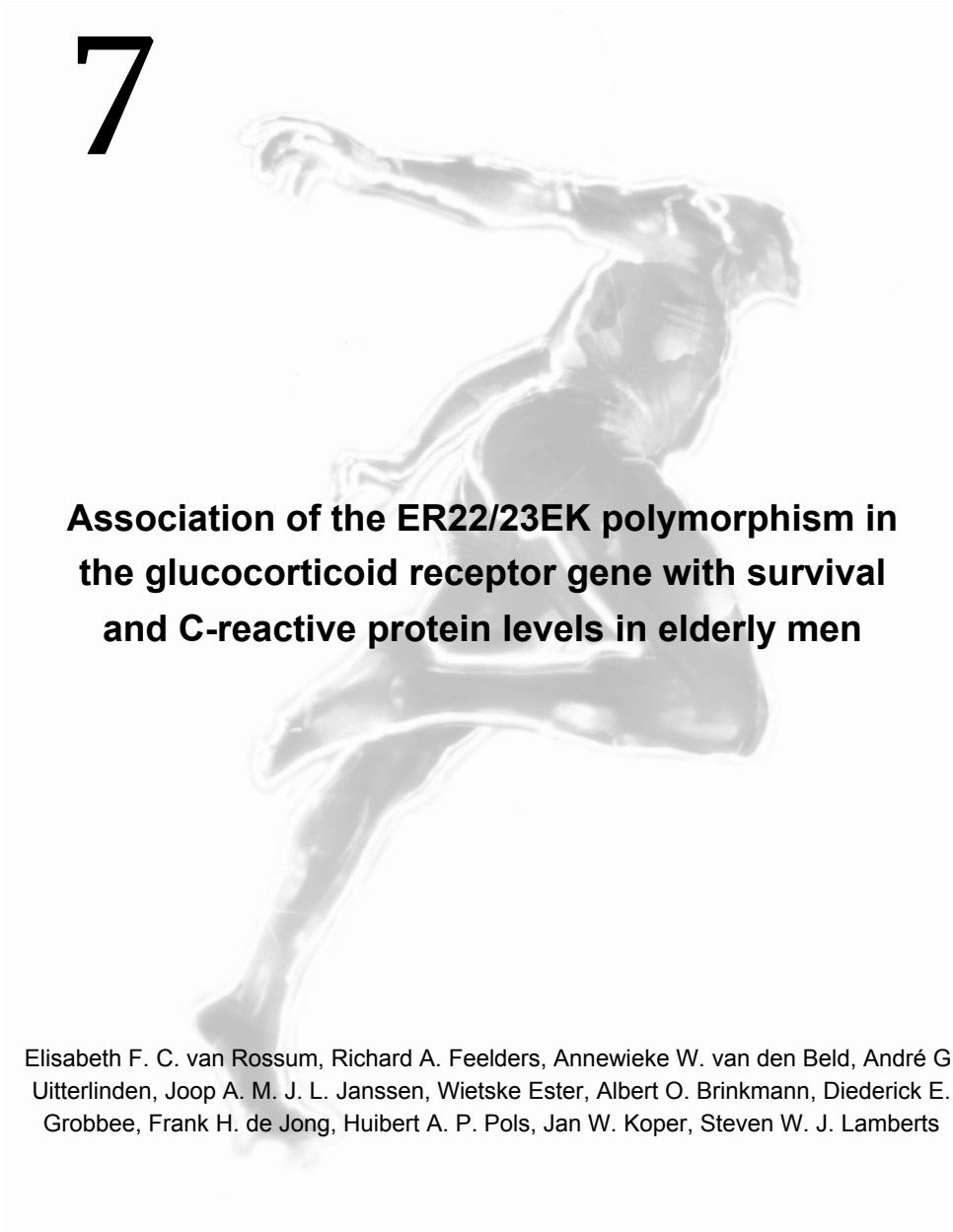

Association of the ER22/23EK polymorphism in the glucocorticoid receptor gene with survival and C-reactive protein levels in elderly men

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Abstract

Purpose: we recently demonstrated that a polymorphism in codons 22 and 23 of the glucocorticoid receptor gene is associated with relative glucocorticoid resistance, greater insulin sensitivity, and lower total and low-density lipoprotein cholesterol levels. In the present study, we investigated whether the ER22/23EK polymorphism is associated with survival, cholesterol levels, and two predictors of mortality: serum C-reactive protein (CRP) and interleukin 6 (IL-6) levels. Methods: we studied 402 men (mean (± SD) age, 77.8 ± 3.6 years). CRP was measured by a highly sensitive method using a latex-enhanced immunonephelometric assay. IL-6 was determined by a commercially available immulite assay. Results: after a follow-up of 4 years, 73 (19%) of 381 noncarriers died, while none of the 21 ER22/23EK carriers had died (P = 0.03). CRP levels were about 50% lower in ER22/23EK carriers (P = 0.01). There were no differences in IL-6 levels. Conclusion: carriers of the ER22/23EK polymorphism have better survival than noncarriers, as well as lower CRP levels.

Introduction

Most of the effects of glucocorticoids are mediated by the glucocorticoid receptor [1]. Recently, we demonstrated that a polymorphism in codons 22 and 23 of the glucocorticoid receptor gene (GAGAGG (GluArg, or ER) → GAAAAG (GluLys, or EK)) is associated with relative glucocorticoid resistance [2]. This ER22/23EK variant was also associated with greater insulin sensitivity and lower total and low-density lipoprotein (LDL) cholesterol levels. Furthermore, we found that the number of ER22/23EK carriers was significantly higher in the older half of the sample, suggesting that the polymorphism had a beneficial effect on survival.

The ER22/23EK variant might also affect the inflammatory response, and elevated levels of two inflammatory markers — CRP and IL-6 — are associated with mortality in the elderly [3]. CRP is an independent risk factor for cardiovascular events [4-7]. IL-6 stimulates the synthesis of CRP, as well as other acute phase proteins in the liver, and is elevated in patients with unstable angina or heart failure [8-11]. Therefore, we investigated whether CRP and IL-6 levels, as well as survival, were associated with the ER22/23EK polymorphism of the glucocorticoid receptor gene.

Methods

Subjects

We recruited 402 men, aged 73 years or older, by a letter that was sent to the oldest men in Zoetermeer, The Netherlands. Subjects were eligible to participate if they were physically and mentally able to visit the study center independently. No additional health-related criteria were used. Medications taken for more than 6 months were recorded. Data on vital status of the participants and causes of death during 4-year follow-up were obtained by contacting the participants' general practitioners. Before the start of the study, which received the approval of the Medical Ethics Committee of the Erasmus Medical Center, all subjects had given their written informed consent to participate.

Measurements

Weight and height were measured, and the body mass index (kg/m^2) was calculated. Blood pressure was measured in sitting position at the right upper arm with a random-zero sphygmomanometer. Total fat mass, trunk fat mass, and lean body mass were measured using dual-energy X-ray absorptiometry (Lunar Corp., Madison, Wisconsin) [12]. Quality assurance, including calibration, was performed every morning, using the standard provided by the manufacturer. Levels of total and high-density lipoprotein (HDL) cholesterol and triglycerides were measured using standard laboratory methods; LDL cholesterol levels were calculated. CRP was measured with a highly sensitive method using a latex-enhanced immunonephelometric

assay on a BN II analyser (Dade Behring, Liederbach, Germany). IL-6 was determined by a commercially available immulite assay (Diagnostic Products Corporation, Los Angeles, California), using aliquots of undiluted sera tested against an absolute IL-6 standard preparation. Based on these standard curves, the concentrations of IL-6 were calculated by the software provided. Cortisol was measured using a radioimmunoassay (Diagnostic System Laboratories, Webster, Texas). Lower extremity function or physical performance was assessed with measurements of standing balance, walking speed, and ability to rise from a chair [13]. A summary performance scale, which ranged from 0 (worst) to 12 (best), was created by summing these scores. Satisfaction in performing activities of daily living was assessed by using a self-administered questionnaire [14]. All items are evaluated on a 4-point scale; higher scores denote greater impairment. At baseline, a 21-item medical history was obtained by a structured questionnaire, according to the following groups: musculoskeletal impairments (including arthritis and fractures); cardiovascular impairments (including symptoms or treatment of angina pectoris, heart failure, hypertension, arrythmia, myocardial infarction, cerebrovascular accident, and shortness of breath); prostate problems (hyperplasia and cancer); other malignancies; endocrine disorders (diabetes mellitus and thyroid disease); and other conditions (dizziness and disturbed vision that impair mobility). A physical examination was performed. None of the participants was being treated for systemic infectious, inflammatory, or malignant disorders at the time of enrollment. Glucocorticoid receptor genotypes were determined by restriction fragment length polymorphism analysis [15]. For confirmation, we reanalyzed all 21 heterozygous samples and 10 wild-type samples, and found identical genotypes.

Statistical Analysis

Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, Illinois). Differences between the ER22/23EK carriers and the noncarriers were adjusted for age and, if necessary, for body mass index or smoking and tested by analysis of covariance using the general linear model procedure. Bonferroni post hoc tests were used to adjust for multiple comparisons. If dependent variables were not normally distributed, logarithmic transformations were applied to normalize them or nonparametric tests (Mann-Whitney *U* test) were used. Continuous variables are reported as mean ± SD or median with the interquartile range. Survival was analyzed using the Kaplan-Meier procedure and log-rank test. To study the association between CRP levels and mortality, CRP levels were divided in two groups (high vs. low, based on the median value). Cox proportional hazards models were used to analyze this relation, adjusting for genotype, diabetes, and health status. Correlations between CRP levels and parameters of body composition and cortisol levels were calculated using Spearman's correlation. $P < 0.05$ was considered statistically significant.

Results

Of the 402 men, 21 (5%) were heterozygous for the ER22/23EK polymorphism. No homozygotes were found. There were no significant differences in age, smoking status, measures of body composition, or lipids between the carriers and noncarriers (Table 1).

Mortality

Of the 381 noncarriers, 73 (19%) died during the 4 years of follow-up, while none of the 21 ER22/23EK carriers had died (Figure 1, $P = 0.03$). Causes of mortality included cardiovascular disease (40%, n = 29), cancer (11%, n = 8), pneumonia (7%, n = 5), cerebrovascular accident (5%, n = 4), miscellaneous (cachexia, infections, pulmonary emphysema: 7%, n = 5), and unknown (30%, n = 22). Men with lower CRP levels (less than the median) had significantly better survival (13% (27/201) died) than those with higher CRP levels (24% (47/201) died; hazard ratio = 1.8; 95% confidence interval: 1.1 to 2.9). Additional analyses that adjusted for genotype, diabetes, or general health status (as physical performance, activities of daily life scores, and morbidity data) did not change these results.

Anthropometric, Metabolic, and Inflammatory Parameters in ER22/23EK Carriers and Noncarriers

ER22/23EK carriers had significantly lower CRP levels than noncarriers (Table 1). Lean body mass tended to be higher in ER22/23Ek-cariers compared to noncarriers; however, this did not reach statistical significance after correction for height. CRP levels correlated significantly with body mass index ($r = 0.16$, $P = 0.002$), total fat mass ($r = 0.15$, $P = 0.003$), and trunk fat mass ($r = 0.16$, $P = 0.001$), but not with lean body mass ($r = 0.02$, $P = 0.63$). Early morning cortisol levels did not correlate with CRP levels ($r = 0.04$, $P = 0.48$) or genotype ($P = 0.81$). We found no differences in body mass index, blood pressure, or levels of HDL cholesterol, triglycerides, or IL-6 between ER22/23EK carriers and noncarriers (Table 1).

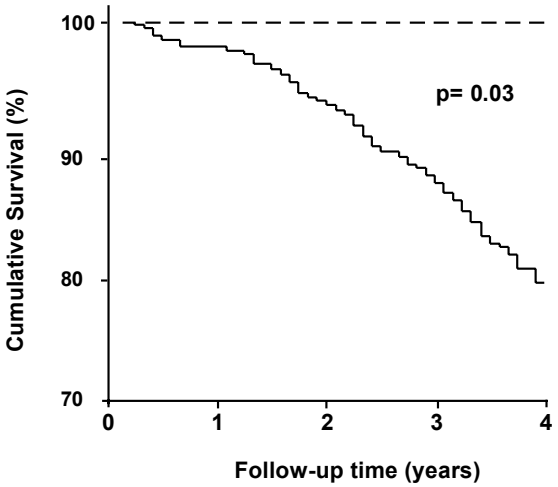

Figure 1: *Kaplan-Meier survival curves by glucocorticoid receptor genotype. ER22/23EK carriers had significantly better survival than noncarriers. The solid line denotes noncarriers; the dotted line denotes ER22/23EK carriers.*

Table 1: Baseline Characteristics of Noncarriers and Carriers of the ER22/23EK Polymorphism among 402 Elderly Men*

Characteristic	Noncarriers (n = 381)	ER22/23EK Carriers (n = 21)	P
	Number (%), Mean ± SD, or Median (Interquartile Range)		
Age (years)	77.7 ± 3.6	78.3 ± 3.6	0.46
Smokers	65 (17)	5 (24)	0.43
Body mass index (kg/m^2)	25.6 ± 4.3	25.9 ± 3.1	0.75
Lean mass (kg)	51.7 ± 5.6	52.8 ± 5.2	0.18†
Fat mass (kg)	21.1 ± 5.7	21.2 ± 6.6	0.89†
Trunk fat mass (kg)	10.6 ± 2.6	10.6 ± 2.9	0.98
Systolic blood pressure (mm Hg)	156 ± 24	158 ± 29	0.67‡
Diastolic blood pressure (mm Hg)	84 ± 11	86 ± 13	0.49‡
Total cholesterol (mmol/L)§	5.8 ± 1.1	5.4 ± 1.0	0.14‡
LDL cholesterol (mmol/L)§	3.8 ± 1.0	3.5 ± 0.9	0.22‡
HDL cholesterol (mmol/L)§	1.3 ± 0.4	1.3 ± 0.3	0.36‡
Triglycerides (mmol/L)§	1.4 ± 0.8	1.3 ± 0.7	0.75‡
C-reactive protein (mg/L)§	4.0 ± 9.1	2.0 ± 2.6	0.01
Interleukin 6 (pg/mL)	19.1 ± 11.1	19.9 ± 9.2	0.47
Diabetes	30 (8)	3 (14)	0.31
Activities of daily life (points)	9 (8-12)	9 (8-11)	0.88
Physical performance (points)	9 (7-10)	9 (7-11)	0.54
Number of chronic diseases	3 (1-5)	3 (1-5)	0.78

*All parameters were adjusted for age, and CRP and IL-6 values were logarithmically transformed. †Lean mass and fat mass were also adjusted for height. ‡Blood pressures and lipids were adjusted for BMI. §To convert total, HDL, and LDL cholesterol levels from mmol/L to mg/dL, multiply by 38.67**;** for triglyceride levels, multiply by 89.15. To convert CRP levels from mg/L to mg/dL, divide by 10. HDL = high-density lipoprotein; LDL = low-density lipoprotein.

Discussion

In this population-based study involving elderly men, we found that carriers of the ER22/23EK polymorphism of the glucorticoid receptor gene had better 4-year survival. This is in line with our previous findings [2], in which we observed that the proportion of ER22/23EK carriers was significantly greater in the older half of the study sample. The ER22/23EK carriers also had significantly lower CRP levels, but no association was found with anthropometric parameters or IL-6 levels. Since CRP level predicts total and cardiovascular mortality in the elderly [16], it might underlie the effect of the ER22/23EK polymorphism on survival. However, it is unclear whether CRP is a cause of atherosclerosis or whether it reflects the degree of vascular damage [17]. We previously demonstrated that carriers of the ER22/23EK polymorphism are relatively resistant to the effects of glucocorticoids, and have lower fasting insulin and cholesterol levels [2]. Perhaps the lower CRP levels we observed in ER22/23EK carriers are related to, and possibly even due to, having less atherosclerosis.

Greater body mass index, and fat mass in particular, are related to higher levels of IL-6 and CRP [18-22]. We found that CRP level correlated positively with body mass index and fat mass in these elderly men, but there was no association between IL-6 and these parameters. We did not observe differences in BMI or fatmas by genotype. ER22/23EK-carriers had a slightly (but not significantly) higher lean body mass. We hypothesized that a greater lean body mass, which might also result in greater insulin sensitivity, may also be a factor in the better cardiovascular health status of ER22/23EK-carriers, thereby contributing to lower CRP levels. In another study, we found associations of the ER22/23EK polymorphism with body composition in young adults, specifically in male carriers aged 36 with a average of 5 kg more lean mass and in female carriers with tendencies towards less fatmass. Muscle mass is an important determinant of insulin sensitivity and can thus contribute to a better metabolic profile. We believe that body composition could play an important role in the relation of the ER22/23EK polymorphism with both survival and CRP levels.

IL-6 is a potent stimulator of CRP in the liver and acts synergistically with glucocorticoids to induce the synthesis of other acute phase proteins by the liver. In addition, glucocorticoids inhibit the production of IL-6 when administered in pharmacological amounts or when present at high levels [23,24]. However, in physiological circumstances, the stimulatory effect of glucocorticoids on IL-6 is minor or even absent [23]. The variation in basal cortisol levels in carriers and noncarriers of the ER22/23EK polymorphism is likely to be within the physiological range because cortisol levels did not differ significantly by genotype. However, in general, the biological response of a target cell to a hormone is determined by several factors, including the concentration of the hormone, the concentration of receptors, and the affinity of the hormone-receptor interaction. For example, glucocorticoids also upregulate the IL-6 receptor [25], by which they can influence the biological effects of IL-6 . The ER22/23EK

polymorphism, which is associated with relative resistance to the effects of glucocorticoids, might result in a lesser degree of upregulation of the IL-6 receptor. Thus, although there were no differences in circulating IL-6 concentrations, the ER22/23EK polymorphism might result in a decreased stimulation of CRP production. In addition, CRP can be synthesized by adipocytes without mediation of IL-6 [26].

Thus, the association between ER22/23EK polymorphism and mortality might be due to factors other than CRP. We reported an association between the ER22/23EK polymorphism and greater insulin sensitivity, as well as lower total and LDL cholesterol levels in a population-based sample that had a mean age of 67 years. Although we observed a similar pattern in the present study, we did not find statistically significant differences in cholesterol levels, perhaps because the mean age in the current study was more than 10 years older; selection of surviving participants with relatively low cholesterol levels may have occurred.

In this study, and in our previous study [2], there was no correlation between ER22/23EK polymorphism and early morning cortisol levels. However, we previously reported that the carrier genotype is associated with glucocorticoid resistance, as manifest by a decreased response to the dexamethasone suppression test [2]. These data suggest that there may be beneficial metabolic effects, resulting in better survival, due to subtle lifelong glucocorticoid resistance. In the present study, we found that the ER22/23EK polymorphism of the glucocorticoid receptor gene was associated with lower CRP levels and better survival in elderly men.

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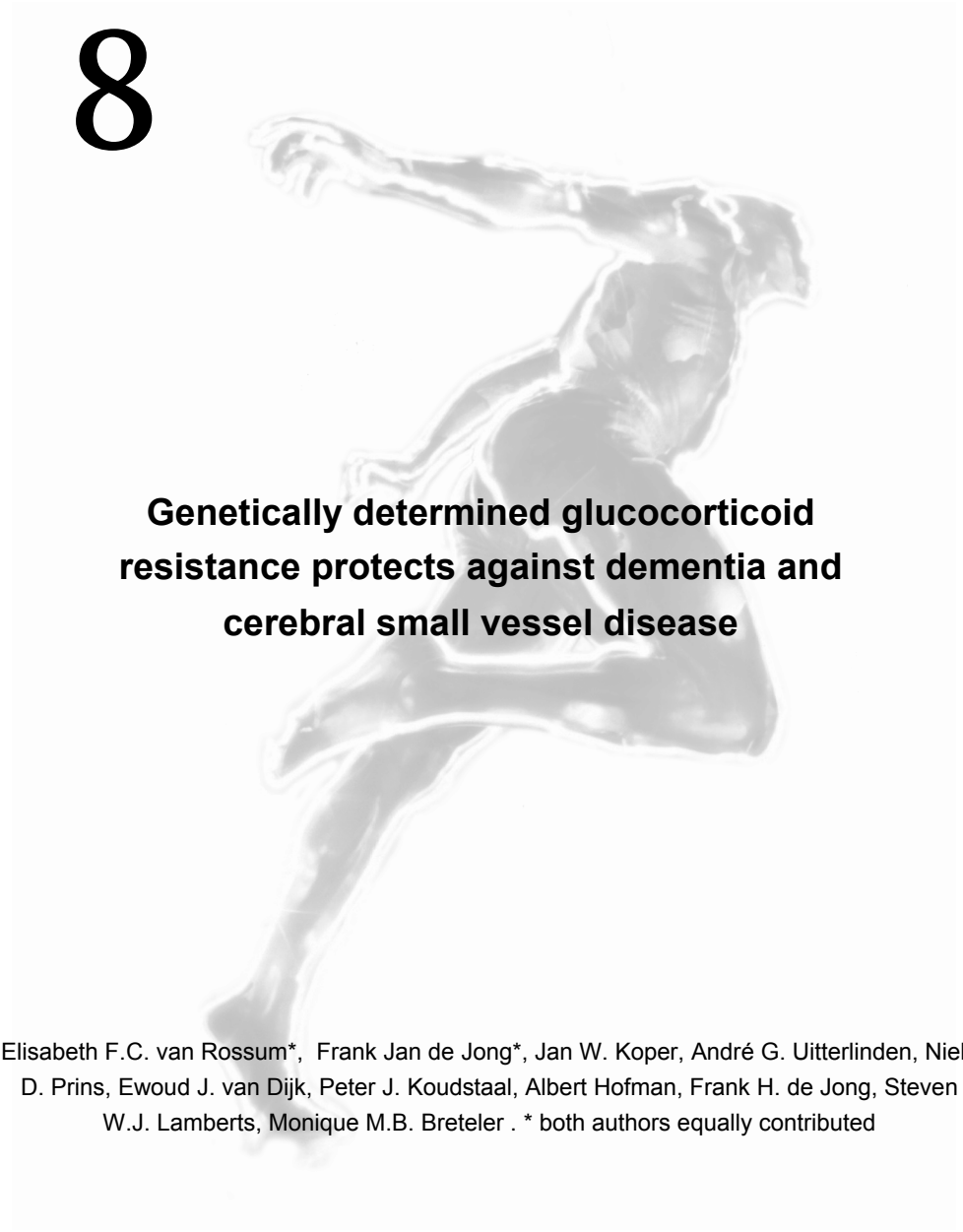

Genetically determined glucocorticoid resistance protects against dementia and cerebral small vessel disease

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Abstract

Context: Glucocorticoids are essential for proper brain functioning. Increased levels of cortisol, related to stress and possibly also to aging, have been associated with cognitive impairment and dementia. The effects of glucocorticoids are mediated by the glucocorticoid receptor (GR). A functional polymorphism (ER22/23EK) of the GR gene (NR3C1) is associated with relative glucocorticoid resistance and a healthy metabolic profile. Objective: We investigated whether the ER22/23EK polymorphism is associated with dementia and structural brain abnormalities.
Design and Setting: 6034 elderly from the Rotterdam Study were screened for dementia during a mean follow-up of 5.8 years. In addition, in 1011 elderly of the Rotterdam Scan Study we investigated the association of this polymorphism with structural brain abnormalities on MRI.
Main Outcome Measures: prevalent and incident dementia, cognitive function (memory and psychomotor speed) in non-demented participants, cerebral white matter lesions, hippocampal and amygdalar volumes. Results: The ER22/23EK polymorphism was negatively associated with the risk of developing dementia. Also at baseline dementia was less prevalent in ER22/23EK-carriers compared to noncarriers (86% risk reduction). In addition, the presence of cerebral white matter lesions and brain infarctions, as well as the risk of progression of white matter lesions was decreased in ER22/23EK-carriers. No association was found with atrophy of the medial temporal lobe on MRI. Among non-demented participants, ER22/23EK-carriers had a better performance on psychomotor speed tests than non-carriers, but no differences were found in memory function between genotypes. Conclusions: Our results suggest a protective effect of the ER22/23EK polymorphism on the risk of dementia and cerebral small vessel disease.

Introduction

Glucocorticoids have a wide variety of effects on peripheral organs, as well as on brain physiology. [1] The glucocorticoid receptor (GR) is the major factor in the mediation of the effects of cortisol. Sensitivity to glucocorticoids between individuals is highly variable, whereas the intra-individual sensitivity is rather stable, suggesting a genetic factor determining sensitivity to glucocorticoids. [2] In this context the classical syndrome of glucocorticoid resistance as a result of a mutation in the glucocorticoid receptor is an example of how important this receptor is in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis. [3,4]

In a study performed in healthy elderly we found that also within the normal population some individuals are relatively glucocorticoid resistant as observed by a diminished suppression in the dexamethasone suppression test. [5] In a subgroup from the Rotterdam Study, a prospective population-based cohort study among elderly, we found several polymorphisms of the *GR* gene.[5] One of these polymorphisms consists of two linked single nucleotide mutations (GAGAGG→GAAAAG) in codons 22 and 23 in exon 2. The first mutation is silent, both codons code for glutamic acid (E). The second mutation, results in a change from arginine (R) to lysine (K). [5] We found that carriers of this ER22/23EK polymorphism were significantly more resistant to the effects of glucocorticoids than noncarriers.[6] We found these ER22/23EK-carriers to have also a better insulin sensitivity and lower cholesterol levels. [6] In a separate population of elderly men we found the ER22/23EK polymorphism to be associated with longevity, as well as lower C-reactive protein levels, possibly reflecting a beneficial cardiovascular profile. [7] In addition, we observed that ER22/23EK-carriers have a sex-specific beneficial body composition at young adult age.[8] All these effects can be explained by their subtle glucocorticoid insensitivity. Recently, the molecular mechanism by which the ER22/23EK polymorphism led to decreased sensitivity to glucocorticoids was elucidated .[9]

No data have been reported concerning the role of the ER22/23EK polymorphism in relation to cognitive function. HPA-axis overactivity, which is related to stress leads to increased levels of cortisol,[10] has been associated with cognitive impairment and dementia.[11,12] In longitudinal studies in both Alzheimer's disease (AD) patients and healthy elderly, higher plasma cortisol levels led to a more rapid decline in cognitive function over time.[12-14] Furthermore, HPA-axis overactivity is related to an increased vascular risk, including hypertension and obesity.[15,16] Increasing evidence suggests that cerebrovascular pathology is important in the etiology and clinical course of dementia and AD.[17,18] In this context, white matter lesions and brain infarctions on MRI, indicative of small vessel disease, have also been associated with cognitive function.[19,20]

We hypothesized a protective effect of the ER22/23EK polymorphism with respect to risk of dementia. Therefore, we investigated the relationship between this polymorphism and the risk of dementia and cognitive performance in the Rotterdam Study. We further studied the

relationship between this polymorphism and both cognitive function and structural abnormalities of the brain on MRI in the Rotterdam Scan Study. The effects on brain structures could be either direct (less harmful cortisol effects due to relative glucocorticoid resistance), or indirect (due to a better metabolic status). In order to study direct cortisol effects on the brain, we tested memory function and measured hippocampal and amygdalar volumes on MRI, which have been shown to be directly affected by increased cortisol levels.[21-23] Indirect effects of a relative cortisol resistance were studied by psychomotor speed tests, as well as cerebral white matter lesions on MRI, which are related to vascular disease.[24]

Methods

Study Design

The Rotterdam Study is a population-based, prospective cohort study designed to study the frequency and determinants of chronic diseases in the elderly.[25] All inhabitants of Ommoord, a district of Rotterdam, the Netherlands, aged 55 years and over including those living in institutions were invited, of whom 7983 gave their written informed consent and participated in the study (response 78%). At baseline, 7,528 subjects were screened for dementia.[26] Of these, 483 were diagnosed to be demented. The cohort at risk of dementia thus comprised 7,045 subjects. Two follow-up examinations took place in 1993-1994 and 1997-1999. The total cohort was further continuously monitored for mortality and major morbidity. Follow-up for dementia was virtually complete (99.9%).

The Rotterdam Scan Study is a prospective cohort study designed to investigate determinants and consequences of brain abnormalities on MRI in the elderly.[27] Between 1995 and 1996 participants were randomly selected from the Rotterdam Study and the Zoetermeer study, another ongoing prospective cohort study in The Netherlands, after stratification by sex and age in 5-year age groups. Elderly with MRI contraindications or dementia at baseline were excluded. Complete information including a cerebral MRI scan was obtained in 1077 participants (response 63%). A total of 951 participants, who were eligible for a second MRI examination, were re-invited in 1999 to 2000 of whom 668 participated (response rate 70%).
Both studies have been approved by the Medical Ethics Committee of Erasmus Medical Center, The Netherlands.

Dementia diagnosis

Case-finding and diagnostic procedures for dementia and AD have been described previously[28] and were equal for both studies. Both at baseline and follow-up examinations, a stepwise procedure was used. First, subjects were cognitively screened with the Mini-Mental State Examination (MMSE)[29] and the Geriatric Mental State (GMS) schedule organic level.[30] Second, if subjects scored below 26 on the MMSE or above 0 on the GMS organic level, the Cambridge

Examination of Mental Disorders in the Elderly (CAMDEX),[31] including an informant interview, was administered. Finally, subjects suspected of having dementia were further examined by a neurologist, a neuropsychologist and, if possible, had magnetic resonance imaging of the brain. In addition, continuous monitoring of the cohort for incident dementia cases took place through computerized linkage between the study database and computerized medical records from general practitioners and through surveillance of Regional Institute for Outpatient Mental Health Care reports.[28] Dementia diagnoses were based on DSM-III-R criteria, AD and vascular dementia diagnoses were subsequently based on the NINCDS-ADRDA and the NINDS-AIREN criteria respectively.[32-34] Final diagnoses were made based on all existing information by an expert panel including the neurologist, neuropsychologist and research physician.

Neuropsychological testing

In addition to the MMSE, which was administered in both studies, participants in the Rotterdam Scan Study underwent more detailed neuropsychological testing at baseline (1995-1996) including an abbreviated Stroop test, the Letter-Digit Substitution task (a modified version of the Symbol Digit Modalities Test), a verbal fluency test, a Paper-and-Pencil Memory Scanning Task and a 15-word verbal learning test (based on Rey's recall of words).[19] From these tests we constructed compound scores for psychomotor speed, memory performance, and global cognitive function by transforming individual test scores into standardized Z-scores.[19]

MRI procedures

Within the Rotterdam Scan Study, cranial MRI scanning was performed in all participants with 1.5-Tesla scanners at two study centers (Gyroscan, Philips NT, Best, The Netherlands or VISION MR, Siemens, Erlangen, Germany) using standard T1, T2 and proton-density weighted MR sequences. MRI acquisition parameters have been described.[19] For the 563 participants of the Rotterdam Study a custom-made double contrast 3D half-Fourier acquisition single-shot turbo spin echo (HASTE) sequence was added for volumetric assessments of the hippocampus and amygdala. In 1999 to 2000 all second MRI scans were made with the VISION MR scanner using the same sequences. All scan assessments were done by raters blinded to any clinical information related to the participants.

Generalized brain atrophy. Generalized brain atrophy was scored on T1-weighted images. Subcortical atrophy was measured by the ventricle-to-brain ratio (average of assessments at three locations, range 0.21 to 0.45). Using reference scans cortical atrophy was rated on a semiquantitative scale (based on the size of gyri and sulci at five locations: 0 (no cortical atrophy) to 3 (severe cortical atrophy). The atrophy score for the sum of all regions ranged from 0 to 15.[35]

Hippocampal and amygdalar volumes. Hippocampal and amygdalar volumes were measured on coronal slices (1.5 mm, no interslice gap) reconstructed from the HASTE sequence to be

perpendicular to the long axis of the hippocampus, as previously described.[36] Briefly, the left and right hippocampus and amygdala were manually traced on each slice by means of a mouse driven pointer and volumes (ml) were calculated by summing the areas multiplied by slice thickness. Total hippocampal or amygdalar volume was calculated by summing the left and right hippocampal or amygdalar volume. Midsagittal area (cm^2) was measured by tracing the inner skull to obtain a proxy for intracranial volume.[36] Head size differences across individuals were corrected for by dividing the uncorrected volumes by the subject's calculated head size area and subsequently multiplying this ratio by the average head size area (men and women separately).[37]

White matter lesions. At baseline, white matter lesions were assessed in all participants of the Rotterdam Scan Study (n=1077) and were considered present if visible as hyperintense on proton density and T2-weighted images, without prominent hypointensity on T1-weighted images and scored in periventricular (range 0-9) and subcortical white matter regions (approximated volume, range 0 to 29.5 ml) on the proton density scans.[19] After 3 years, 668 participants underwent repeated MRI scanning. Change in periventricular and subcortical white matter lesion severity was rated with a semiquantitative scale, and progression was rated as no, minor or marked progression.[38]

Brain infarctions. We defined brain infarcts as focal hyperintensities on T2-weighted images, 3 mm in size or larger. Proton-density scans were used to distinguish infarcts from dilated perivascular spaces. Hyperintensities in the white matter also had to have corresponding prominent hypointensities on T1-weighted images, in order to distinguish them from cerebral white matter lesions.[20]

Genetic analysis

At baseline peripheral venous blood samples were drawn and genomic DNA was isolated from whole blood using standard techniques. Genotyping was performed by allelic discrimination using TaqMan Universal PCR master mix (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), primers (forward: 5'-TCCAAAGAATCATTAACTCCTGGTAGA-3'and reverse:5'-GCTCCTCCTCTTAGGGTTTTATAGAAG-3') and probes (Applied Biosystems) and a Taqman ABI Prism 7700 Sequence Detection System (Applied Biosystems). Used probes were 5'-FAM-ACATCTCCC**C**T**C**TCCTGAGCAAGC-3' and 5'-VIC- ACATCTCCC**T**T**T**TCCTGAGCAAGCA-3' (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60° C and optimized concentrations for primers and probes of 400 nmol/L and 100 nmol/L, respectively. We re-analyzed genotypes in 100 samples by PCR-RFLP analysis using the *MnlI* restriction enzyme (New England Biolabs, Leusden, The Netherlands) and a digestion of 1 hour at 37 °C and found identical genotypes.

Assessment of covariates

Covariates were assessed similarly in both studies. Body mass index (BMI) was calculated as weight divided by the square of height. Blood pressure was measured twice on the right arm with a random zero sphygmomanometer. We used the average of these two measurements. Diabetes mellitus was defined present if participants reported use of oral antidiabetic treatment or insulin, or if a random serum glucose level exceeded 11.1 mmol/l at baseline. Smoking habits were assessed with a structured questionnaire. Serum total cholesterol levels were determined using an automated enzymatic procedure. A history of stroke at baseline was obtained through self-report and by checking medical records. Once subjects enter the Rotterdam Study, they are continuously monitored for major events through automated linkage of the study database with files from general practitioners and the municipality. Also nursery home physician's files are scrutinized. For reported events, additional information (including brain images) is obtained from hospital records. An experienced stroke neurologist reviewed all available information on all possible strokes and transient ischemic attacks to diagnose and categorize types of stroke. Apolipoprotein-E (APOE) genotyping was performed on coded DNA samples without knowledge of the diagnosis. The PCR product was digested with the restriction enzyme *HhaI*, and fragments were separated by electrophoresis.[39]

Data analysis

First, we examined the relation between the ER22/23EK polymorphism and dementia within the Rotterdam Study. The likelihood for ER22/23EK-carriers of being demented at baseline was assessed by means of logistic regression. The prospective relation with incident dementia was assessed with Cox proportional hazard models. Follow-up time was calculated from baseline until death, diagnosis of dementia, or end of follow-up, whichever came first. Age at onset of dementia was determined as age at diagnosis. Linear regression analysis was used to study the association between the polymorphism and MMSE in both the Rotterdam Study and the Rotterdam Scan Study, and to analyze cognitive functioning in more detail using compound scores for neuropsychological tests in the Rotterdam Scan Study. Since the ER22/23EK polymorphism has been associated with absence, rather than presence of disease, compound scores were further analyzed using logistic regression after dichotomization at the median level to compare relatively bad performers (below the median) with good performers (above the median).

Differences between the distribution of the ER22/23EK polymorphism and structural abnormalities on MRI were studied in the Rotterdam Scan Study. Both measures of brain atrophy and white matter lesions were analyzed using analysis of covariance (ANCOVA). We used logistic regression to investigate the possible association with the presence of a brain infarct on the baseline scan, and - since we hypothesized that the ER22/23EK polymorphism is associated with absence rather than presence of white matter lesions - with white matter

lesions dichotomized according to presence or absence of these lesions at baseline. Progression of white matter lesions at follow-up was also analyzed according to presence or absence of progression of lesions (no versus any progression). Due to the low number of participants with marked progression (approximately 10% in both periventricular and subcortical regions), minor and marked progression were not analyzed separately. The limited number of incident infarcts also precluded a separate analysis on incident infarcts on MRI.

All analyses were adjusted for age and sex. To elucidate whether associations might be explained by vascular intermediates, analyses were repeated after additional adjustments for hypertension, body mass index, diabetes, cholesterol levels, as well as smoking status and exclusion of subjects with a history of stroke at baseline. In addition, subjects with a stroke preceding a dementia diagnosis were censored at the date of stroke diagnosis in the analyses on dementia incidence (and follow-up time was then calculated from baseline until diagnosis of stroke). The analyses on progression of white matter lesions where also repeated after adjustment for baseline white matter lesions. In all analyses, heterozygous and homozygous carriers were analyzed together as carriers of the ER22/23EK polymorphism. All analyses were performed using SPSS statistical software version 11 (SPSS Inc., Chicago, Illinois).

Results

Baseline characteristics for both study samples are shown in table 1. GR genotypes were present for 6034 participants in the Rotterdam Study. We identified 389 heterozygous ER22/23EK-carriers (6.5%) and 7 homozygous ER22/23EK-carriers (0.1%) in this population. At baseline, data on dementia were present in a total of 5990 participants of whom 395 carried the ER22/23EK polymorphism. In the Rotterdam Scan Study, GR genotypes were present for 1011 participants (78 (7.7%) heterozygous and 1 (0.1%) homozygous ER22/23EK-carriers respectively). Genotype frequencies of both study populations were in Hardy-Weinberg equilibrium.

Dementia

In the Rotterdam Study genotype frequencies of the ER22/23EK allele were 6.8% in the non-demented and 1.2% in the demented at baseline. Two out of 172 participants with dementia (both AD) had one ER22/23EK allele. After adjustment for age and sex, the frequency of the ER22/23EK allele was significantly lower (more than 80%), in both dementia and AD patients compared to non-demented subjects (table 2). After exclusion of those demented at baseline, the cohort was followed for incident dementia. During 38.763 person-years of follow-up (mean (SD) 5.8 (1.6) years) 329 participants developed dementia, of whom 243 had AD.

Table 1: Baseline characteristics of the study populations*

	Rotterdam Study	Rotterdam Scan Study†
Number of study participants	6034	1011
Age (years)	69.3 (9.0)	72.3 (7.4)
Gender (% female)	59.7	51.4
Smoking (% current)	22.2	17.2
Diabetes Mellitus (%)	10.1	7.1
Systolic blood pressure (mmHg)	139.3 (22.2)	147.6 (21.6)
Diastolic blood pressure (mmHg)	73.7 (11.4)	78.9 (11.8)
Body mass index (kg/m^2)	26.3 (3.7)	26.6 (3.6)
Total cholesterol (mmol/l)	6.6 (1.2)	5.9 (1.0)
ER22/23EK carriers (%)	6.6	7.8

* Values represent means (standard deviation) or percentages (%)

† Overlap with Rotterdam Study n=515 participants

Sixteen participants with one ER22/23EK allele developed dementia during follow-up (of whom 12 had AD and 3 vascular dementia), whereas none of the homozygous carriers did). Genotype frequency of the ER22/23EK allele was 6.9 % in the non-demented and 4.9 % in the demented participants respectively The ER22/23EK polymorphism was negatively associated with the risk of developing dementia. Risk for both overall dementia and AD was nearly 40% lower in carriers of the ER22/23EK-allele (table 2). The association remained unchanged after adjustment for potential cardiovascular intermediates or APOE4 genotype and exclusion of strokes at baseline or censorship of incident strokes.

Table 2: Frequencies of the ER22/23EK polymorphism and risk of dementia*

	Noncarriers	ER22/23EK	OR (95%CI)	P
Prevalent dementia				
Overall dementia (n/N*)	170/5595 (3.0%)	2/395 (0.5%)	0.14 (0.03; 0.59)	0.01
			HR (95%CI)	
Incident dementia				
Overall dementia	313/5425 (5.8%)	16/393 (4.1%)	0.63 (0.38; 1.04)	0.07

OR, odds ratio, 95% CI, 95% confidence interval, HR, hazard ratio, p, p-values for carriers of the ER22/23EK allele (non-carriers are reference). * number of cases / total number in the analysis

Cognitive function

After exclusion of those who were demented, MMSE scores at baseline where slightly higher in carriers of the ER22/23EK polymorphism, though differences were non-significant in both studies. The adjusted difference between ER22/23EK-carriers and noncarriers was 0.05 point (95% Confidence Interval (CI): -0.13; 0.23) in the Rotterdam Study and 0.26 points (95% CI: -0.24; 0.77) within the Rotterdam Scan Study. ER22/23EK-carriers also had higher scores on the compound scores for memory performance, psychomotor speed and global cognitive function in the Rotterdam Scan Study. The average differences were not statistically significant (adjusted differences (95% CI) in Z-score for memory performance, psychomotor speed and global cognitive function: 0.06 (-0.13; 0.25), 0.13 (-0.04; 0.30) and 0.10 (-0.04; 0.24) respectively), but carriers were more than twice as likely to score better on tests of psychomotor speed when dichotomized at the median level (table 3).

Table 3: ER22/23EK polymorphism and cognitive performance*

Compound score	Odds Ratio (95% CI)	P
Memory	1.02 (0.63; 1.67)	0.93
Speed	2.24 (1.30; 3.89)	0.004
Overall cognitive function	1.18 (0.70; 1.99)	0.54

* Values represent Odds Ratios (95% confidence intervals (CI)) for carriers of the ER22/23EK allele to have better cognitive performance (noncarriers are reference).

Structural brain abnormalities on MRI

In the Rotterdam Scan Study, no significant differences were observed between carriers and noncarriers of the ER22/23EK polymorphism with respect to any of the measures of brain atrophy on MRI, including hippocampal (p=0.49) and amygdalar volume (p=0.50), cortical atrophy (p=0.50) and ventricle-to-brain ratio (p=0.43).

Table 4. ER22/23EK polymorphism and white matter lesions on MRI*

	White matter lesions			
	Periventricular		Subcortical	
	OR (95% CI)	P	OR (95% CI)	P
Presence	0.47 (0.28; 0.80)	0.005	0.40 (0.21; 0.79)	0.008
Progression	1.02 (0.47; 2.20)	0.96	0.34 (0.14; 0.84)	0.02

*Values represent odds ratios (OR (95% confidence interval)) for presence and progression of white matter lesions, noncarriers are reference.

Carriers of the ER22/23EK polymorphism had slightly less severe white matter lesions at baseline, but differences were not statistically significant. The adjusted differences for ER22/23EK-carriers compared to noncarriers were –0.38 points (95% CI: -0.84; 0.07 points) and –0.18 ml (95% CI: -0.81; 0.45 ml) for periventricular and subcortical white matter lesions respectively. However, when presence or absence of white matter lesions was compared, ER22/23EK carriers were less than half as likely to have these lesions than noncarriers (48% for periventricular and 40% for subcortical white matter lesions respectively) (table 4). Similarly, ER22/23EK carriers were less likely to have a brain infarct on their MRI scan (age and sex adjusted OR 0.76 (95% CI: 0.43; 1.32). In addition, ER22/23EK-carriers had almost 70% less progression of subcortical white matter lesions (table 4). This association remained unchanged after adjustment for baseline subcortical white matter lesions (OR for any progression: 0.26 (95% CI: 0.09; 0.73). Progression of periventricular white matter lesions was not different between ER22/23EK-carriers and noncarriers (table 4).

Discussion

In the Rotterdam Study, a prospective population-based study in the elderly, we found that the functional ER22/23EK polymorphism of the *GR* gene is associated with a nearly 40% risk reduction of incident dementia during a follow-up period of almost 6 years, supported by significantly less (86%) prevalent dementia at baseline. In addition, we observed in the Rotterdam Scan Study that ER22/23EK-carriers performed better on psychomotor speed tests and less often had periventricular and subcortical white matter lesions or brain infarctions on

MRI. Progression of subcortical white matter lesions was also significantly reduced in ER22/23EK-carriers. Interestingly, several indicators of cerebrovascular pathology did not significantly differ between genotypes when analyzed continuously. However, when we divided the participants according to presence or absence of white matter lesions, or good or bad psychomotor speed performance, we found highly significant differences. This supports our hypothesis that this polymorphism is related to healthy conditions rather than to pathological conditions. In both studies we did not observe associations with memory function in non-demented subjects.

There are several possible explanations for the protective effects on the brain we observed in carriers of the ER22/23EK polymorphism. First, the ER22/23EK polymorphism has previously been shown to be associated with a relative resistance to glucocorticoids with respect to the negative feedback in normal individuals.[6] The regulation of glucocorticoid production is modulated by a negative feedback mechanism of glucocorticoids at the level of the hypothalamus and pituitary, which is mediated by the GR. High levels of cortisol have been shown to impair cognitive function, and low cortisol levels after dexamethasone, indicative of negative feedback function, are related to cognitive decline.[11,12,14] Also, atrophy of the hippocampus is facilitated by cortisol.[22,23] Cortisol levels have been shown to be increased in both vascular dementia and Alzheimer's disease.[11,12] Thus, the lower risk on dementia and white matter lesions in ER22/23EK-carriers might be related to a decreased direct effect of cortisol on the brain, which is possibly mediated by a relative insensitive GR. However, in the present study we did not observe any differences in hippocampal or amygdalar volumes. Atrophy of the hippocampus and amygdala is associated with decreased memory function and is an early marker of Alzheimer's disease.[21,37] In accordance, in the Rotterdam Study, we did not observe differences in memory function between non-demented carriers and noncarriers. Also in the Rotterdam Scan Study, we found only an association with psychomotor speed function, but not with memory function. This could be explained by the presence of the mineralocorticoid receptor (MR), of which the expression in the brain is restricted to the hippocampal and amygdalar regions.[40] Glucocorticoids can also bind with high affinity to the MR.[41] In rat brain, it has been shown that glucocorticoids activate only MR when present in low concentrations (during basal conditions), and higher concentrations (during stress conditions) activate both MR and GR.[41] Thus, glucocorticoid balance in the hippocampus and amygdala in basal state seems to be mainly regulated by the MR. Therefore, the beneficial effects of a subtle resistance of GR might be less in these brain regions. However, we have to be careful with the interpretation of these studies of corticosteroid receptors in the brain, because most data are from animal studies and it is not known whether they can be extrapolated to the human brain.

An alternative, second explanation for the beneficial cerebral effects of the ER22/23EK polymorphism, is that this GR variant has previously been associated with lower

total cholesterol and LDL-cholesterol levels.[6] Atherosclerosis has been associated with a higher prevalence of Alzheimer's disease [42] and cognitive decline.[43] In addition, periventricular, and to a lesser extent subcortical white matter lesions were previously shown to be associated with atherosclerosis.[44,45] Since ER22/23EK-carriers had less often white matter lesions, as well as less progression of these lesions, the underlying mechanism might be a better vascular status in ER22/23EK-carriers. This is supported by our finding of better psychomotor speed scores in carriers of this polymorphism, since psychomotor speed performance is associated with the presence of cerebral white matter lesions.[19] However, adjustment for markers of atherosclerotic disease did not change our results. On the other hand, a limitation of our study is that only cholesterol levels at high age are available. Since midlife cholesterol concentrations in particular have been shown to be related to an increased risk of dementia,[42] we cannot rule out the possibility that atherosclerosis underlies the beneficial effects of this polymorphism on the brain.

A third mechanism, which possibly relates to the effects of the ER22/23EK variant might be through the glucose/insulin metabolism. In this context, the ER22/23EK polymorphism has previously been associated with a better insulin sensitivity in the elderly.[6] Increased serum insulin concentrations and diabetes have been shown to be associated with decreased cognitive function and dementia.[46,47] The effects of changes in insulin homeostasis on the brain can be either direct or via the process of atherosclerosis.[47,48]

A fourth factor in the protective effects of the ER22/23EK variant on the brain might be inflammation.[49,50] In a population of 402 elderly men, the ER22/23EK polymorphism was associated with longevity and lower C-reactive protein (CRP) levels.[7] In a recent study [50] higher CRP levels were associated with an increased risk on dementia. However, CRP levels might also be a reflection of cerebrovascular status.

The molecular mechanism for the reduced glucocorticoid sensitivity as a result of the ER22/23EK polymorphism, has recently been elucidated. Previously, Yudt and Cidlowski described alternative start-codon usage (AUG-1 or AUG-27) in the GR gene. [51] The use of AUG-27 results in a slightly shorter but transcriptionally more active form of the GR-protein, termed GR-B.[51] We have observed that the nucleotide changes involved result in alterations in the secondary structure of the GR mRNA, which may shift the balance of start codon usage from AUG-27 to AUG-1. As a result we observed a reduction in the formation of the shorter, transciptionally more active GR-B synthesis.[9]

We conclude from these prospective large-scale population studies that the ER22/23EK variant of the *GR* gene is associated with a lower risk on the development of dementia during a follow-up period of nearly 6 years, as well as less prevalent dementia in the elderly. Furthermore, this polymorphism is associated with a better cognitive performance with respect to psychomotor speed, and lower risk of periventricular and subcortical white matter lesions, as well as less progression of subcortical lesions. Knowledge of the factors involved in

the development of dementia is of great clinical relevance, because eventually it could lead to intervention or even prevention of this frequently occurring disease. Our data suggest that the mechanisms underlying the better cognitive functioning in ER22/23EK-carriers might be a relative resistance to glucocorticoids and, at least in part, less cerebral vascular disease.

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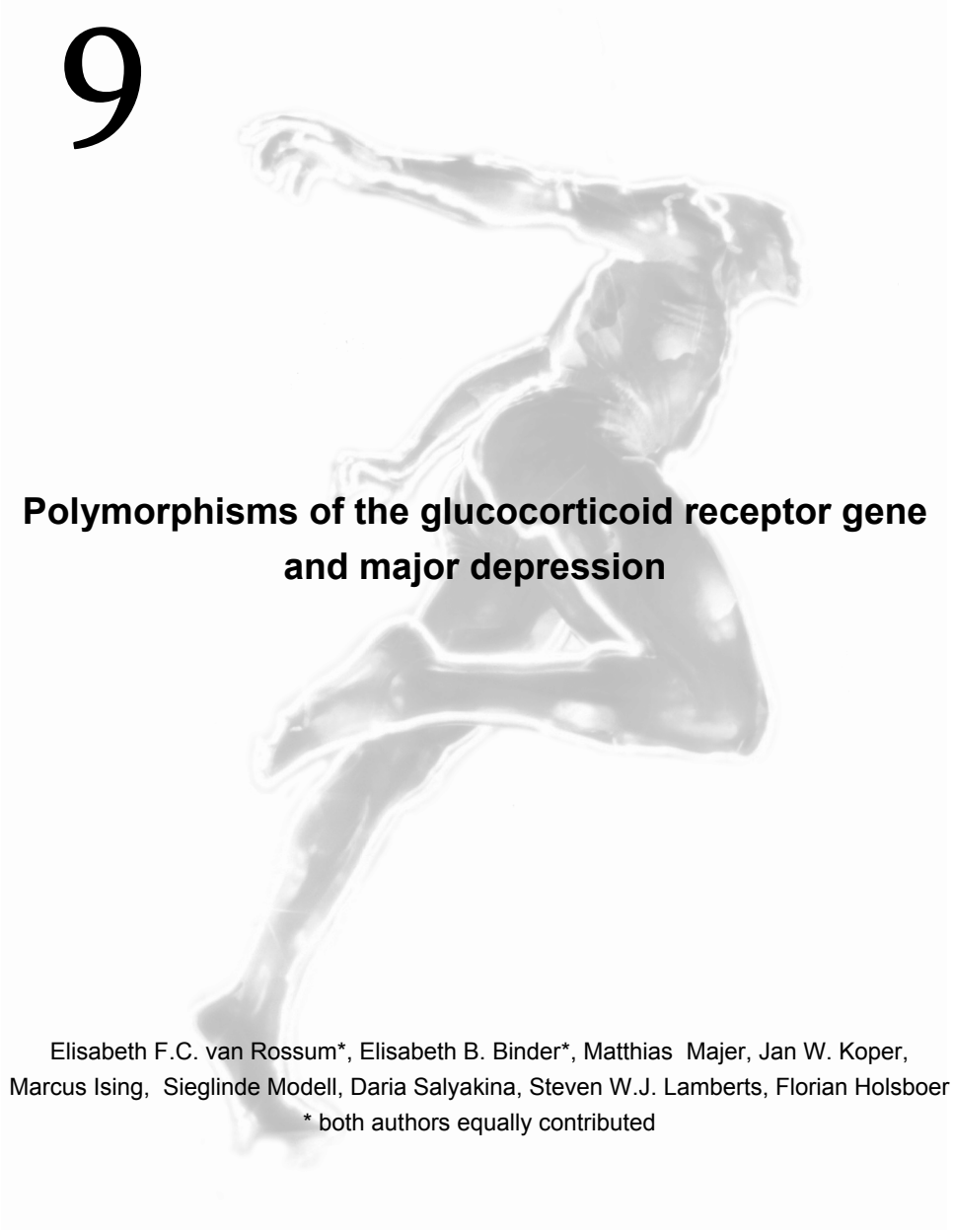

Polymorphisms of the glucocorticoid receptor gene and major depression

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Submitted

Abstract

Background: The most consistent biological finding in patients with depression is a hyperactivity of the hypothalamic-pituitary-adrenal (HPA)-axis which may be caused by impaired glucocorticoid signaling. Glucocorticoids mainly act through the glucocorticoid receptor (GR) for which several functional polymorphisms have been described. Two variants of the *GR* gene (N363S and *BclI*) are associated with increased glucocorticoid sensitivity while a third, consisting of two linked mutations (ER22/23EK) is associated with relative resistance to glucocorticoids. Methods: We studied whether the susceptibility to develop a depression is related to these polymorphisms by comparing depressive inpatients (n=490) and healthy controls (n=496). Among depressed patients, we also investigated the relation between the GR variants and dysregulation of the HPA-axis, as reflected by ACTH and cortisol responses to the dexamethasone suppression and the combined dexamethasone suppression/CRH-stimulation test (Dex-CRH test), the clinical response to antidepressive treatment and cognitive functioning. Results: Homozygous carriers of the *BclI* polymorphism ($p = 0.01$) as well as ER22/23EK carriers ($p = 0.04$) had an increased risk of developing a major depressive episode compared to noncarriers. We did not find associations of these GR polymorphisms with functional HPA-axis measures in depressed patients. Carriers of the ER22/23EK polymorphism, however, showed a significantly faster clinical response to antidepressant therapy, as well as a trend towards better cognitive functioning during depression.

Conclusions: The *BclI* GG and ER22/23EK polymorphisms were associated with susceptibility to develop major depression. In addition, the ER22/23EK polymorphism is associated with a faster clinical response to antidepressant treatment. These findings support the notion that variants of the GR gene may play a role in the pathophysiology of a major depression and can contribute to the variability of antidepressant response.

Introduction

A concatenation of data implicates hyperactivity of the hypothalamic-pituitary adrenal (HPA)-axis in the pathogenesis of depression and its normalization as a necessary predecessor of clinical response to antidepressant drugs [1]. An impaired signaling pathway via corticosteroid activated glucocorticoid receptors (GR), leading to an impaired negative feedback regulation and thus to partial glucocorticoid resistance appears to cause this hyperactivity [2]. In depressed patients this is reflected by basal hypercortisolemia and cortisol escape from dexamethasone suppression[3], as well as an increased ACTH and cortisol release in the combined dexamethasone suppression/CRH-stimulation test (Dex-CRH test) [4-6]. Animal data also support this hypothesis as a transgenic mouse strain, expressing an antisense mRNA directed against the GR gene leading to impaired GR expression, displays not only neuroendocrine abnormalities similar to depressed patients, but also depression-like behavioral changes [7 8]. In response to antidepressant treatment, partial GR resistance is restored [9]. Clinical studies have shown that a resolution of the HPA-axis hyperactivity and GR insensitivity precedes clinical improvement to antidepressant drugs in patients [10-13]. In addition, *in vivo* as well as *in vitro* studies suggest that antidepressants not only increase GR gene expression but also the sensitivity to glucocorticoid activation [2 14-17]. Increased GR activation may also promote depressive symptoms. In Cushing's disease, characterized by severely increased cortisol levels, symptoms of depression frequently occur [18]. In addition, glucocorticoids exert a positive feedback on CRH expression in limbic regions such the amygdala [19 20]. Increased CRH neurotransmission in limbic regions has been associated with increased depression-like symptomatology [20]. Therefore, not only glucocorticoid resistance seems to be related to depression, but also enhanced GR effects in limbic brain regions may contribute to the development of depression. This makes the GR a prime candidate gene forassociations with susceptibility for depressive disorders as well as an altered clinical response to antidepressant drugs.

Numerous studies describe a high heritability of depressive disorders [21] and genetic factors leading to a changed GR sensitivity may therefore alter the susceptibility to depression and the response to antidepressant drugs. In addition, functional variants in this gene are also likely to affect the outcome of neuroendocrine tests, such as the Dex-CRH test, and neuropsychological tests in depressed patients. Neuropsychological deficits are frequently observed in depressed patients and may be related to increased HPA-axis activity. Longterm increases of circulating glucocorticoids have been associated with cognitive impairment, mostly of hippocampal [22-24] but also of ventral prefrontal functions such as the functions of the anterior cingulate which include divided attention [25]. In a recent study, we observed that the severity of neuropsychological deficits in a test for divided attention at admission was predictive for response to antidepressant treatments [26].

In previous studies, we reported the characterization of several functionally relevant polymorphisms of the *GR* gene: the first is located in codon 363 (exon 2), which results in an asparagine (N) to serine (S) amino acid change (N363S). A second polymorphism involves a *BclI* restriction site in intron 2, and is a C to G nucleotide change, 646 bp downstream from exon 2 (*BclI*). These two *GR* gene polymorphisms were associated with a hypersensitivity to glucocorticoids, as observed by an increased response to the ACTH- and cortisol-suppressive effects of low dose dexamethasone [27] [28] and a metabolic profile suggestive of GR hypersensitivity [29]. In previous studies it has been shown that the N363S polymorphism is associated with obesity [30-33], while others could not detect any relationship with body composition [34] [35]. With respect to the *BclI* polymorphism also associations with obesity have been reported [36-38]. In two healthy elderly populations the *BclI* polymorphism was associated with lower body mass index, due a lower amount of lean mass, which also may be explained by an increased sensitivity to glucocorticoids [28]. At present, it is not known what the effects of these two polymorphisms are on the brain.

Another polymorphism of the *GR* gene, also located in exon 2, consists of two linked nucleotide changes in codons 22 and 23 (GAG AGG → GAA AAG). The first nucleotide change in codon 22 is silent, both coding for glutamic acid (E), and the second results in an amino acid change from arginine (R) to lysine (K). This ER22/23EK polymorphism was associated with a decreased response to the administration of 1 mg dexamethasone, suggestive of GR resistance [39]. In line with this, carriers of the ER22/23EK variant also had a better insulin sensitivity and lower total cholesterol and low-density lipoprotein cholesterol, as well as a sex-specific beneficial body composition [40]. In addition, the ER22/23EK polymorphism was associated with lower C-reactive protein levels and longevity [41], as well as with a reduced risk on cerebral white matter lesions and dementia[42]. Recently, the molecular mechanism for the reduced glucocorticoid sensitivity as a result of the ER22/23EK polymorphism has been elucidated. We observed a change in the balance between two previously reported [43] translational variants of the GR protein (GR-A and GR-B). As a result of the ER22/23EK polymorphism we found a reduction in the formation of the shorter, transciptionally more active GR-B synthesis and an increase of the longer, transciptionally less active GR-A[44], which could explain the relative glucocorticoid resistance.

In the present study we investigated the role of these three functional polymorphisms of the *GR* gene in: 1. the susceptibility to develop a depressive episode, 2. the disturbance of the HPA-axis regulation which often accompanies depression using the Dex/CRH test, 3. the response to antidepressive treatment, and 4. cognitive functioning with respect to divided attention.

Methods

Patients

490 patients admitted to our psychiatric hospital for treatment of a depressive disorder presenting with a unipolar depressive episode (85.6 %), bipolar disorder (13.2 %) or dysthymia (1.2%) as their primary psychiatric diagnoses were recruited for the study. Patients were included in the study within 1-3 days of admission to our hospital and the diagnosis was ascertained by trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria. Depressive disorders due to a medical or neurological condition were exclusion criteria. Ethnicity was recorded using a self-report sheet for perceived nationality, mother language and ethnicity of the subject itself and all 4 grandparents. All included patients were Caucasian and 92 % of German origin. The study has been approved by the local ethics committee. Written informed consent was obtained from all subjects.

Psychopathology and definition of response to antidepressant drug treatment

In 367 patients severity of psychopathology at admission was assessed using the 21 items Hamilton Depression Rating Scale (HAM-D) by trained raters, including residents in psychiatry and psychologists. Ratings were performed within 3 days of admission and then in weekly intervals until discharge. All patients were treated at doctor's choice with antidepressant drugs within a few days of admission. For all patients plasma concentration of antidepressant medication was monitored to assure clinically efficient drug levels. For the analysis of medication related effects, patients were grouped according to their primary medication within the first five weeks of treatment in patients having received selective serotonin reuptake inhibitors (SSRI), tricyclic antidepressant (TCA) and mirtazapine, a drug targeting serotonergic and noradrenergic receptors.

The Dex-CRH test

The Dex-CRH test was performed as described in detail by Heuser et al [4]. Patients were administered the test within the first ten days of admission (n= 342) and the last ten days of discharge (n = 258). Briefly, patients were pre-treated with 1.5 mg of dexamethasone per os at 23:00. The following day a venous catheter was placed at 14:30 and blood was drawn at 15:00, 15:30, 15:45, 16:00 and 16:15 into tubes containing EDTA and trasylol (Bayer Inc., Germany). At 15:02 100 µg of human CRH (Ferring Inc., Kiel, Germany) was administered intravenously. For the dexamethasone suppression test (DST), morning cortisol was assessed at 8:00 the morning before and after 1.5 mg dexamethasone. Hormone assays for the Dex-CRH test and the DST were identical to those described in detail by Zobel et al [45]. Briefly, for the measurement of plasma cortisol concentrations, a radioimmunoassay (RIA) kit from ICN Biomedicals, Carson, CA was used with a detection limit of 0.3 ng/ml. For plasma ACTH

concentrations an immunometric assay without extraction (Nichols Institute, San Juan Capistrano, CA) was used, with a detection limit of 4.0 pg/ml.

Neuropsychological testing

Neuropsychological testing was performed within the first 10 days after admission (n = 196) and within the last week before discharge (n = 168). The ability to divide attention is mostly assessed by dual-task paradigms, therefore we used the sub-test *divided attention* of the TAP (*Testbatterie zur Aufmerksamkeitsprüfung* (version 1.02b; [46]). This task requires simultaneous attention to acoustic (a series of high and low sounds) and visual (changing crosses on a computer screen) stimuli. Acoustic targets are the repetition of high or low sound, visual targets is any formation of a square composed of four crosses. The subject is required to press as fast as possible a response button. Performance is scored as mean reaction time in trials with target stimuli present.

Controls

496 controls matched for ethnicity (using the same questionnaire as for patients), sex: patients with 58.0 % females and 42.0 % males and controls with 59.2 % females and 40.8% males (X^2 = 0.15, df = 1, p = 0.69) and age: patients with a mean age of 47.57 (SD = 14.5) and controls 46.9 (15.0) (ANOVA. $F_{954,1}$=0.39; p = 0.53) were recruited. Controls were selected randomly from a Munich-based community sample and screened for the presence of anxiety and affective disorders using the Composite International Diagnostic-Screener. Only individuals negative in the screening questions for the above-named disorders were included in the sample. Recruitment of controls was also approved by the local ethics committee and written informed consent was obtained from all subjects.

Genotyping

On enrollment in the study, 40 ml of EDTA blood were drawn from each patient and DNA was extracted from fresh blood using the Puregene® whole blood DNA-extraction kit (Gentra Systems Inc; MN). Allelic discrimination was performed to genotype the subjects, using TaqMan Universal PCR master mix, primers and probes (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands, see also table 1) and a Taqman ABI Prism 7700 Sequence Detection System as previously described [28]. Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60° C and optimized concentrations for primers of 400 nmol/L for each polymorphism. Concentrations of probes we used are listed in table 1. We re-analysed genotypes of all heterozygous and homozygous carriers of the polymorphisms and found identical genotypes.

Statistical analysis

All statistical analyses were performed using SPSS (version 11). All analyses for binary outcomes were performed using exact contingency table analyses. Test for odds ratios and allelic association in the case/control study were performed using tests adapted from Sasieni PD (1997) available on http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. We used three types of response definition commonly used in psychiatric research. Early partial response at two weeks was defined as a greater than 20% decrease of HAM-D scores from the score obtained at admission [47]. Patients with a reduction > 20% from their score at admission were considered as "early responders", while patients whose HAM-D score decreased ≤ 20% from the score at admission were considered as "early non-responders". For both, response at 4 weeks of treatment and response at discharge a reduction of over 50% from HAM-D scores at admission was required to meet the responder criterion at these time points. All patients with a reduction of HAM-D scores equal to or less than 50% were assigned to the group of non-responders. For analysis of genotype-related effects on response we also used a repeated measures ANOVA with weekly HAM_D score from admission to week 4 as the within subject factor and a repeated measures ANCOVA with the HAM-D score on admission as covariate controlling for possible baseline differences. For analysis of genotype-related effects on cortisol and ACTH response to the Dex-CRH test a repeated measures ANOVA with the 5 consecutive plasma cortisol and ACTH values from 15:00 to 16:15 against genotype was used. Other quantitative outcomes were analyzed using a one-way ANOVA. Considering the small n in some subgroups we also analyzed genotype related effects on neuropsychological and also endocrine measures using non-parametric analyses. Individual haplotype assignments for the 3 polymorphisms were determined using SNPHAP. Only haplotype assignments with a remaining uncertainty of less than five percent and haplotypes with a frequency over five percent were included in the analyses. Linkage disequilibrium among the 3 markers was estimated with D' and r2 using Haploview.

Results

Haplotype frequencies and linkage disequilibrium pattern

All SNPs were in Hardy Weinberg Equilibrium in both the case as well as the control group. Haplotype estimation revealed that individuals with haplotypes containing more than one carrier allele were very rare (< 0.3%). Haplotype analysis did therefore not yield any additional information to single SNP analysis. As expected linkage disequilibrium as measured by r*2* is very low among all markers (see Table 2).

Table 2: Haplotype frequencies (total sample) and linkage disequilibirium among GR polymorphisms in D' and r^2.

		D'	r^2
ER22/23EK	N363S	0.06	0.0
ER22/23EK	*Bcl*1	1.0	0.01
N363S	*Bcl*1	1.0	0.03

ER22/23EK	N363S	*Bcl*1	frequency (%)
1	1	1	55.82
1	1	2	36.68
1	2	1	4.40
2	1	1	2.87
2	2	1	0.00
1	2	2	0.00
2	1	2	0.00
2	2	2	0.00

Case-control associations and relation to disease parameters

Significant differences in genotype frequency could be detected between healthy controls and depressive patients for *Bcl*I, genotypic p value = 0.026, allelic p value 0.01, OR = 1.3 (95% CI = 1.063 – 1.598) but not for of the other investigated GR polymorphisms (see table 3 for frequency distribution) when looking at all depressed patients. We then focused our analysis first on patients with unipolar depression and then recurrent unipolar depression. While this did not change the association with Bcl1, ER22/23EK showed a significant case/control association and N363S a trend for such an association with unipolar recurrent depression. For ER22/23EK, the genotype distribution in controls was 476 (95.8%) non-carriers and 21 (4.2%) carriers and in unipolar recurrent depressed patients 169 (92.3%) vs. 14 (7.7%), respectively (allelic p = 0.043, OR = 1.98 (95% CI = 1.009 – 3.084)). For N363S the genotype distribution in controls was 336 (91.3%) non-carriers and 32 (8.7%) carriers and in unipolar recurrent depressed patients 142 (86.4%) vs. 23 (13.6%), respectively (allelic p = 0.11). We could not detect any association of the investigated polymorphisms with age of onset, number of previous depressive episodes nor distribution of unipolar vs. bipolar disorder or psychotic vs. non-psychotic depression. There was also no genotype-dependent difference in the primary class of antidepressant drug treatment in the first five weeks of treatment.

Table 3: Frequencies of three polymorphisms of the glucocorticoid receptor gene in healthy controls and depressive patients. *1 patient homozygous for the carrier allele. §1 control homozygous for carrier allele.

Polymorphism		Healthy controls N (%)	Depressive patients N (%)
ER22/23EK	Non-carriers	476 (95.8 %)	462 (94.3 %)
	Carriers	21 (4.2 %)	28* (5.7 %)
N363S	Non-carriers	336 (91.3 %)	395 (90.8 %)
	Carriers	32§ (8.7 %)	40 (9.2%)
BclI	Non-carriers	163 (43.6 %)	162 (37.0 %)
	Heterozygous carriers	174 (46.5 %)	208 (47.5%)
	Homozygous carriers	37 (9.9%)	68 (15.5%)

Response to antidepressant treatment

Patients carrying the ER22/23EK polymorphism responded more quickly to antidepressant drug treatment. We found a significant association of the ER22/23EK genotype and early responder status after two weeks of treatment ($p = 0.008$). Among patients that were heterozygote for the ER22/23EK polymorphism only 2 were in the early partial non-responder group, while 19 were in early partial responder group (see table 4). This corresponds to an odds ratio of 5.17 (95% CI: 1.18 – 22.58) (allelic test). Using a repeated measures ANOVA for HAM-D scores from admission to week 4 against the ER22/23EK genotype we observed a significant effect of the change in HAM-D score between admission and the first 4 weeks of treatment ($F(4,315)=94.6$; $p< 0.0001$). When controlling for HAMD-D baseline difference on admission we found a significant genotype effect of the ER22/23EK variant on the HAM-D score ($F(1,314)=5.01$; $p=0.026$) (figure 1). Carriers of the ER22/23EK polymorphism remitted (HAM-D score below 10) on average 5 days faster to antidepressant treatment than non-carriers. However, HAM-D scores at admission and at discharge did not differ significantly between carriers and non-carriers of the ER22/23EK polymorphism (HAM-D score at admission, mean (SD) = 25.5 (7.4) for non-carriers and 25.4 (8.0) for ER22/23EK-carriers; HAM-D score at discharge = 9.2 (5.0) for non-carriers and 9.7 (6.7) for ER22/23EK-carriers). No significant associations between response to treatment and the *BclI* or N363S polymorphisms were found.

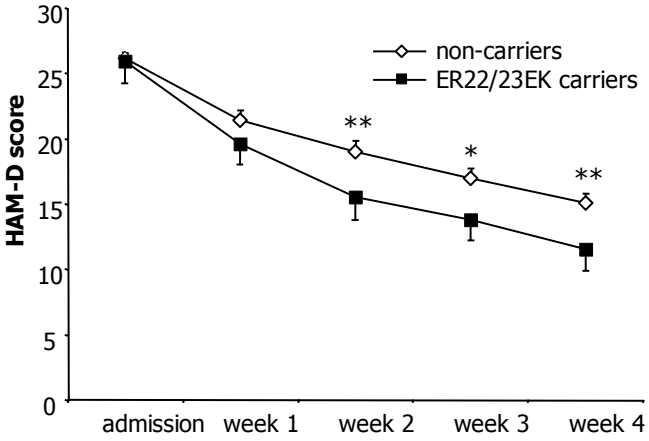

Figure 1: *The response to antidepressant treatment in relation to the ER22/23EK polymorphism of the glucocorticoid receptor gene. ER22/23EK-carriers (black squares) responded faster to treatment than non-carriers (white squares). N = 297 non-carriers and 20 carriers. HAM-D, Hamilton Depression Rating Scale. **p<0.05, *p=0.06*

Neuroendocrine and neuropsychological measures in depressive patients in relation to GR polymorphisms

Using a repeated measures ANOVA, one way ANOVA and non-parametric tests, we found no significant genotype effect on ACTH or cortisol response in the Dex-CRH test at admission, as well as discharge for any of the tested polymorphisms. Homozygous carriers for *BclI* showed a non-significantly higher cortisol response in the Dex-CRH test at admission (p = 0.1) and N363S carriers showed a trend for a higher cortisol response from admission to discharge (repeated measures ANOVA on cortisol area under the curve (AUC) from admission to discharge (p = 0.068). No significant genotype-related differences were found for basal morning cortisol, morning cortisol after DEX and the ratio between these two parameters (suppression ratio). There was a trend for a lower basal morning cortisol in ER22/23EK carriers (p = 0.09). Figures 2A-F depict the Dex-CRH test and the DST at admission and at discharge according to the 3 polymorphisms.

Table 4: Frequencies of the ER22/23EK polymorphism according to early partial response status at 2 weeks of antidepressant treatments.

Polymorphism		Early non-responders N (%)	Early responders N (%)	P
ER22/23EK	non-carriers	122 (98.4 %)	224 (92.2 %)	0.008
	carriers	2 (1.6 %)	19 (7.8 %)	

In order to study whether cognitive functioning in depression is related to GR polymorphisms we assessed parameters of divided attention in all depressive patients. Using a Mann-Whitney U test, we observed a shorter reaction time in the test for divided attention in patients carrying the ER22/23EK polymorphism (N=10) than in patients not carrying this polymorphism (N=186, 667 ± 93 ms vs 726 ± 134 ms, p = 0.08) . At discharge, ER22/23EK-carriers (N=9) also appeared to perform better in this test compared to non-carriers (N=159, 637 ± 108 ms vs 695 ± 112 ms), albeit this difference did not reach statistical significance (p= 0.26). No significant associations of the reaction time in this test could be found for the *BclI* or N363S polymorphisms.

Discussion

Major depression is a disorder with high heritability [21] but individual episodes are frequently triggered by stressful life events, pointing to a role of stress hormones in the etiology of this disease. Indeed, it has been shown that depression is often accompanied by a hyperactivity of the HPA-axis related to an impaired negative feedback regulation of the GR, which normalizes prior to clinical response to antidepressant treatment [1]. Because of their inherent differences in glucocorticoid function, carriers of the ER22/23EK, N363S and Bcl1 polymorphisms could be expected to be more or less prone to develop a major depression. In this study the frequency of the *BclI* GG carriers was significantly higher in cases than in healthy controls. The frequency of ER22/23EK-carriers was not significantly higher in depressive patients compared to controls when tested for all depressive patients. However, after selection of only unipolar, recurrent depressive patients, we observed an association with the ER22/23EK polymorphism, which might be explained by a more genetically determined susceptibility to develop depressive episodes of this severely affected subgroup.

It may seem surprising, that two polymorphisms, which have been associated with opposite effects of GR sensitivity [29] are both related to depression. As mentioned in the introduction, however, increased as well as decreased GR sensitivity could lead to depressive symptoms. Glucocorticoids have been shown to act as positive or negative transcriptional regulator of the neuropeptide CRH in a brain region dependent manner. While the GR exerts a negative feedback on CRH in the hypothalamus, it increases CRH expression in limbic regions such as the amygdala [19 20]. Increased CRH in limbic regions has been linked to depression-like symptoms [1]. Previously, we have shown that heterozygous and homozygous *BclI*-carriers have an increased sensitivity to glucocorticoids with respect to the adrenal negative feedback action [28]. Homozygous *BclI* carriers may thus have an increased positive glucocorticoid feedback on CRH in the limbic system, resulting in an increased production of CRH and thereby depressive symptoms.

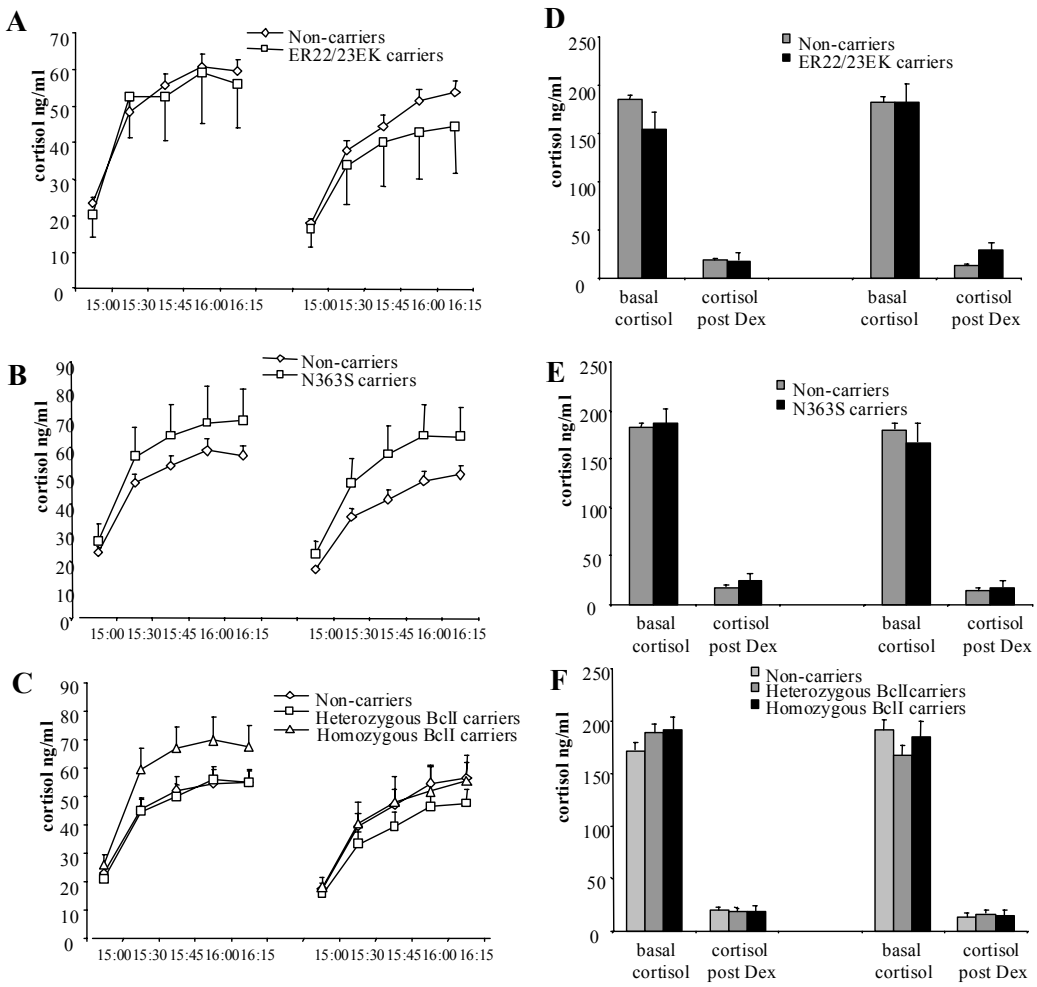

Figure 2: *Panels A-C show the morning cortisol response in the Dex-CRH test at admission (on the left) and at discharge (on the right) according to A: ER22/23EK, B: N363S, C: BclI. 100 µg of CRH were administered at 15:02. Panels D-F show the morning cortisol levels in the DST at admission (on the left) and at discharge (on the right) according to D: ER22/23EK, E: N363S, F: BclI.*

On the other hand, it has been shown that this polymorphism has highly tissue-specific effects, with both increased as well decreased GR sensitivity reported [48 49]. It is therefore still unclear how this polymorphism would affect GR sensitivity in the different brain regions. Increased GR resistance has been described as one of the endocrine hallmarks of depression part of a vicious

circle of HPA-axis dysregulation leading to an upregulation of central CRH and AVP expression [1] [2]. The inherent GR-resistance associated with ER22/23EK carrier status may thus predispose these patients to develop depressive symptoms in response to stressful life events. It has to be noted though, that especially for the 2 relatively rare polymorphisms (ER22/23EK and N363S) the power to detect such association may have been low and replications in much larger cohorts are needed.

In healthy probands, the relative resistance to glucocorticoids associated with the ER22/23EK variant was reflected by a diminished plasma cortisol response to the suppressive effects of an overnight administration of 1 mg of dexamethasone [39]. The relative hypersensitivity of the glucocorticoid receptor associated with the N363S and *BclI* polymorphism was identified by an increased response to the ACTH- and cortisol-suppressive effects of low dose dexamethasone [27] [28]. In the present study, we could not detect consistent effects of these polymorphisms in the Dex-CRH test and the DST administered to depressed patients. This may be explained by the previously described state-dependent GR resistance and increased activity of the ACTH secretagogues CRH and arginine vasopressin (AVP) presumed during an acute depressive episode, leading to increased HPA-axis-responses in all genotypes [1] [50]. These state-dependent effects on HPA-axis regulation subside after remission as reflected by a normalization of the Dex-CRH test performed at discharge in both carriers and non-carriers. In parallel with the findings in healthy probands, one might have expected to see the previously observed endocrine phenotypes in the DST administered at discharge, with the subsiding of the state dependent HPA-axis abnormalities. It has to be noted though that only 60% of the discharged patients show a complete remission of their depressive symptoms (HAM-D score < 10). When the analyses were restricted to only these patients, we still did not observe any genotype-dependent differences in the DST. In addition to a possible lack of power, especially with the rarer polymorphisms, this may be due to several factors impacting the HPA-axis in remitted depressed patients. It has been shown that among remitted patients, those who will relapse within 6 months, already display a marked hyperactivity in the Dex-CRH test [45]. Furthermore, previous depressive episodes and HPA-axis hyperactivity may permanently alter the reactivity of the axis as the activation of the GR has been shown to changes methylation pattern of specific genes [51]. Finally, differences in the doses of dexamethasone used for healthy probands and patients could also account for these differences.

The clinical response to treatment, especially during the first weeks of treatment was significantly faster in ER22/23EK-carriers. This is not likely due to associations of the genotype with differences in disease history or treatment regimen that may influence response to antidepressant treatment as the ER22/23EK variant was not associated with any of the tested parameters related to these variables. Experiments in transgenic mice showed that genetically determined functional GR impairment can be overcome by antidepressant treatment, leading to a normalization of the behavioral and neuroendocrine phenotype [9]. A common *in vitro* effect of

many antidepressant drugs is an increase in GR mRNA levels, as shown in neuronal cells [52], fibroblasts [7] and peripheral blood cells [53]. Also *in vivo* an upregulation of GR expression following antidepressant treatment has been reported in hippocampal regions and the hypothalamus of rats [14-17]. In addition, Pariante et al reported that several types of antidepressants inhibit membrane steroid transporters and thereby stimulate GR-mediated gene transcription and thus enhance the effects of cortisol [54]. Finally, clinical studies have shown that a resolution of the HPA-axis hyperactivity and GR insensitivity precedes clinical improvement to antidepressant drugs [4 10 11 13 55]. ER22/23EK-carriers may benefit more rapidly than non-carriers from an upregulation and increased function of the GR following antidepressant treatment. Due to a chronic state of GR-resistance, parallel regulatory pathways attenuating HPA-function may be more easily modulated by antidepressants in ER22/23EK carriers. One of these parallel regulatory pathways could be the HPA-axis suppressive effects of hippocampal mineralocorticoid receptors (MR). The MR binds glucocorticoids with higher affinity than the GR and activation of hippocampal MR has been shown to exert tonic inhibitory effects on the HPA-axis [56 57]. Following antidepressant treatment, the hippocampal expression of this receptor is upregulated more quickly than GR expression [14-17]. In addition, persistent GR resistance may alter the expression profile of GR-regulating chaperones and co-chaperones. One of them, FKBP5, has been shown to be involved in faster response to antidepressant drugs[58].

Neuropsychological deficits are commonly seen in depressed patients [59]. In the present study, cognitive function was tested by a test for divided attention, that is a task requiring simultaneous attention to acoustic and visual stimuli. Impairment in divided attention has been showed to be related to therapy-resistance, as well as an elevated risk to relapse during a follow-up period of six months [26]. During the acute phase of the depressive episode ER22/23EK-carriers tended to perform better than non-carriers in this test, suggesting that carriers of the ER22/23EK variant may be relatively protected from possibly harmful effects of elevated HPA-axis activity on cognitive function [25]. In addition, at discharge there was a non-significantly better performance by ER22/23EK-carriers in the divided attention test as well. This suggests that either the baseline level of this type of cognitive functioning is higher in ER22/23EK-carriers, or that the deleterious effects of a depressive episode on the brain are sustained longer in non-carriers. Majer et al [26] indicated that the divided attention test could be used as a valuable predictor for the course of depression and control of therapy effectiveness, as a better performance in this test at admission was associated with a faster response to antidepressant drugs [26]. This is in accordance with our observations that the ER22/23EK polymorphism is associated with both a faster response to antidepressant therapy, as well as a slightly better divided attention performance.

The three genetic polymorphisms influencing GR sensitivity in non-depressed subjects, did not show any association with HPA-axis related endocrine outcomes in depressed patients.

Depression-related HPA-axis dysfunction appears to override the inherent GR-polymorphism-related ones, indicating that this system can be regulated at various levels. The *BclI* and the ER22/23EK polymorphism of the GR gene appear to be associated with the development of depression. In future studies this finding needs to be replicated in different and larger populations. The ER22/23EK variant, that has been associated with partial GR resistance in healthy subjects, was also associated with a faster response to antidepressant therapy. This finding is of particular interest with regards to a paper recently published by our group that showed a strong association of polymorphisms within a GR-regulating co-chaperone of hsp90, FKBP5, with response to antidepressant drugs [58]. If this present finding is replicated, this would be the second HPA-axis regulating gene associated with favorable response to antidepressant treatment and may point to HPA-axis regulation as a common final mechanism of action of currently used antidepressants.

In conclusion, this is the first report, demonstrating an association of functional GR polymorphisms with depression and antidepressant response. This further strengthens the hypothesis of a causal involvement of the HPA-axis in the pathogenesis of depression.

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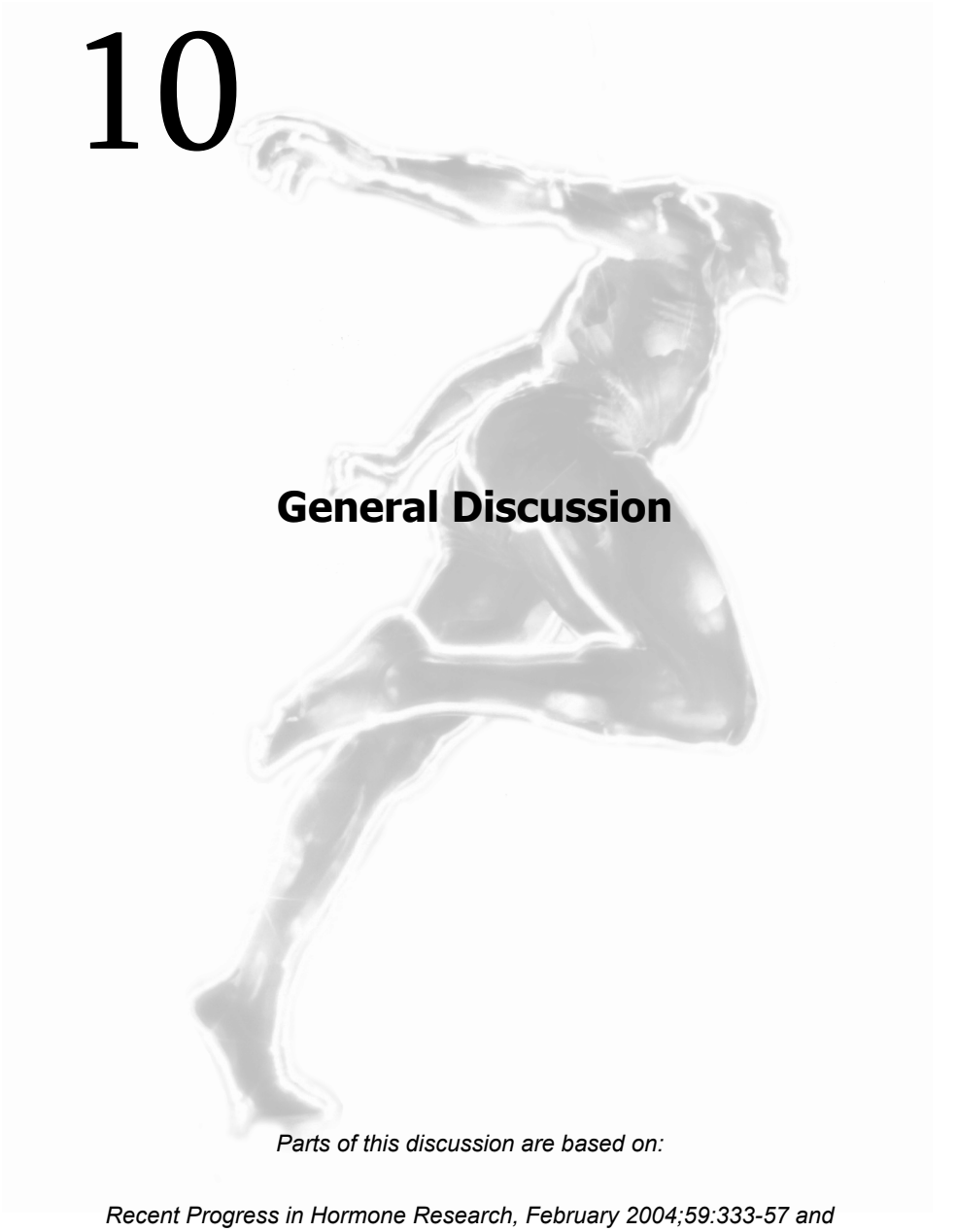


General Discussion

Parts of this discussion are based on:

Recent Progress in Hormone Research, February 2004;59:333-57 and
Trends in Endocrinolgy and Metabolism 2005, in press

In this thesis we focussed on several polymorphisms of the *GR* gene, which are associated with altered glucocorticoid (GC) sensitivity, body composition, metabolic parameters, and cerebral effects. As shown in figure 1 three functional polymorphisms are located in exon 2 (transactivating domain) and intron 2, and one variant is located in exon 9β. This is in contrast to the previously described rare mutations causing the syndrome of GC resistance, which are predominantly located in the ligand-binding domain. Glucocorticoids (GCs) are essential for many regulatory processes in the human body, so a mutation leading to absolute resistance to GCs seems not compatible with life. The previously described patients, carrying a mutation of the *GR* gene, demonstrated a decreased negative feedback at the level of the pituitary gland. This leads to hyperactivation of the HPA-axis. Many of the symptoms, which can be found in patients with GC resistance are the consequence this compensatory increased HPA-axis activity: hyperandrogenism (in particular leading to symptoms in females and children before puberty) and increased mineralocorticoid effects. The latter effects are due to the exposure of the mineralocorticoid receptor to high concentrations of cortisol, which cannot be effectively inactivated by 11β hydroxysteroiddehydrogenase II (11β HSD II).

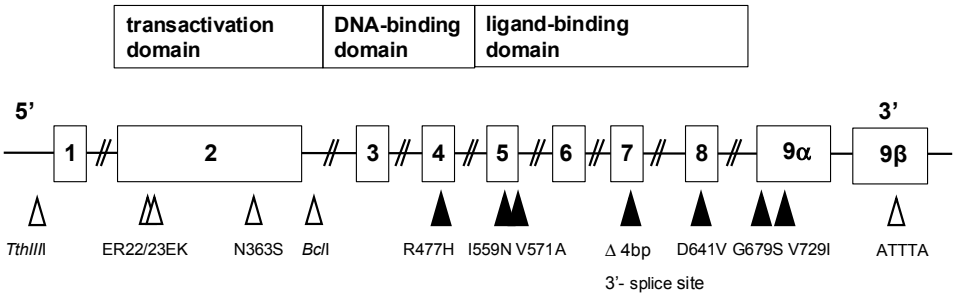


Figure 1: *Schematic overview of the GR gene, showing polymorphisms (white arrows), which have been shown to alter GC sensitivity and are associated with differences in body composition and metabolism, as well as mutations (black arrows) leading to the syndrome of cortisol resistance.*

Polymorphisms, which are common variations at the DNA level occurring in the normal population, have much more subtle effects. However, because of their high frequency in the population their impact may be considerable. In several studies the polymorphisms in the GR gene described here seem to be significantly associated with variations in sensitivity to endogenous GCs within the normal population. Table 1 shows an overview of the four polymorphisms discussed in this thesis and their relation with altered GC sensitivity.

Table 1: Four Polymorphisms of the GR gene, Studied in the Same Population in Relation to Glucocorticoid Sensitivity.

Polymorphism	BclI	N363S	ER22/23EK	TthIIII
N	191	216	202	205
Fasting Cortisol	not different	Not different	not different	not different
Sensitivity to 1 mg DEX	increased	Not different	decreased	not different
Sensitivity to 0.25 mg DEX	increased	Increased	not different	not different

DEX: dexamethasone, not different: no differences between genotype groups of the above mentioned polymorphism, N: number of individuals studied as a sample of the normal elderly population

No associations were found with the TthIIII polymorphism. However, the ER22/23EK variant was found to be linked to the TthIIII polymorphism, and in this respect the associations with GC resistance and beneficial metabolic profile (low insulin and cholesterol levels) were also observed in carriers of both the ER22/23EK and the TthIIII polymorphisms. Table 2 shows the sequence alterations of the two noncoding polymorphisms (TthIIII and BclI) we identified and described in this thesis. Considering the outcomes of the DEX suppressions tests in carriers of the three functional polymorphisms, it seems that the 0.25 mg DEX suppression test is most sensitive to detect hypersensitivity to GCs, while the 1 mg DEX suppression test may be more suitable to detect a relative resistance to GCs (as shown in figure 2 for the polymorphisms described in this thesis).

Table 2: Fragments Length of the BclI and the TthIIII Restriction Fragment Polymorphisms and their corresponding Nucleotide Changes, as well as Allelic Frequencies

RFLP	Length restriction fragment*	Nucleotide change	Allele frequency**
BclI	2.3 kb	C	65 %
	4.5 kb	G	35 %
TthIIII	3.4 kb	C	62 %
	3.8 kb	T	38 %

* Fragments length as described in the literature. After identification of the exact nucleotide change we found that the fragments of the BclI polymorphism were 2.2 kb and 3.9 kb, respectively.** Allele frequency as observed in a subset of subjects from the Rotterdam study, a population-based study in the elderly. RFLP, restriction fragment length polymorphism

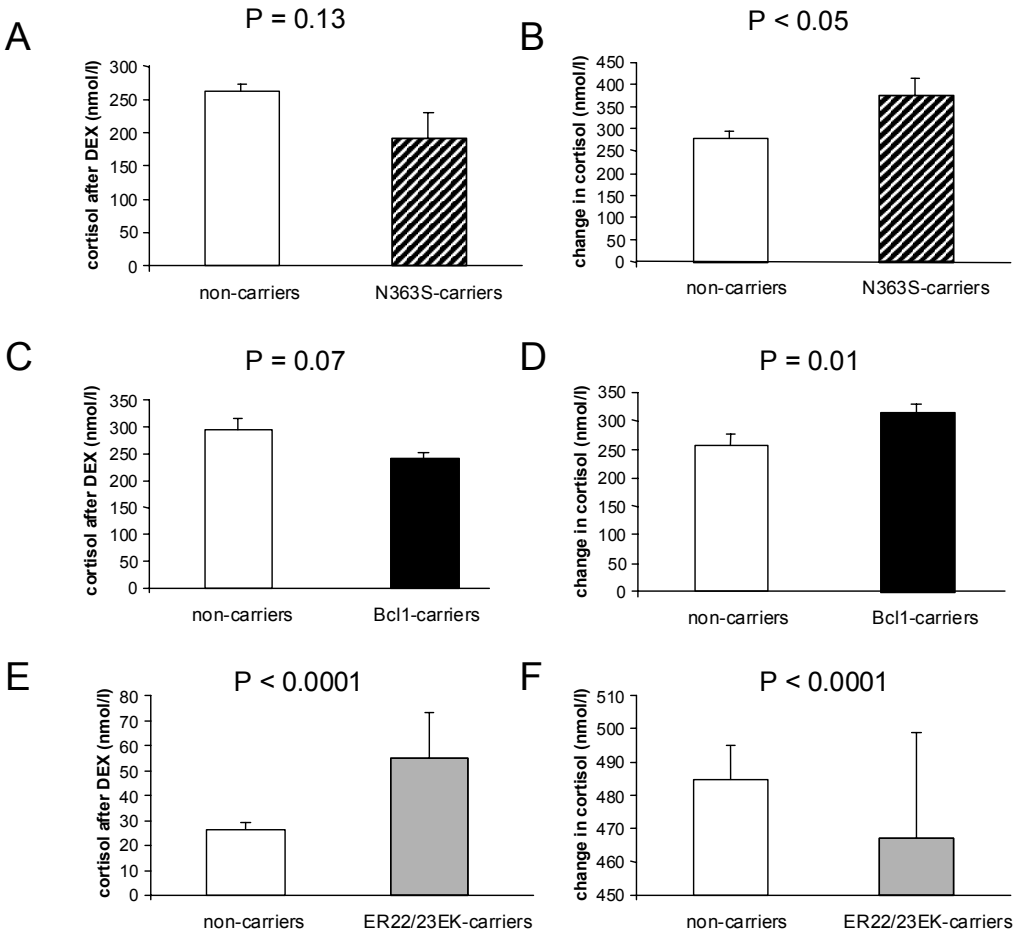


Figure 2: *Cortisol levels (nmol/l) after DEX suppression tests (graphs on the left: **A, C, E**) and absolute change in cortisol (nmol/l) after DEX (graphs on the right: **B, D, F**). Results from a 0.25 mg DST are shown for the N363S and BclI carriers, and data concerning a 1mg DST are shown for the ER22/23EK-carriers. Noncarriers (white bars) were compared to: (**A** and **B**) N363S-carriers (striped bars), lower post-DEX cortisol and greater decrease in N363S-carriers, suggest a hypersensitivity to GCs, (**C** and **D**) heterozygous and homozygous BclI G-allele carriers (black bars), also lower post-DEX cortisol and greater decrease in BclI G-allele carriers, suggesting that BclI G-allele carriers are hypersensitive to GCs, and (**E** and **F**) to ER22/23EK-carriers (vertically striped bars), who had higher cortisol levels after 1 mg DEX and a smaller decrease in cortisol, which suggests that the ER22/23EK variant is associated with a relative resistance to GCs.*

Facts and Fallacies

In the past few years rapid progress in human genome sequence determination [1, 2] has stimulated research concerning the role of polymorphisms in genes, which are possibly involved in the pathogenesis of common diseases. In the past decades linkage analysis has been a widely used study design to investigate the genetic basis of hereditary diseases [3-5]. Linkage studies involve a search for genomic regions with a number of shared alleles higher than expected among affected individuals within a family. Using a wide variety of genetic markers a region can be identified, wherein an allele is present which is predisposing for a certain disease. The identified linked regions can be fine-mapped with additional markers. Linkage analysis is a powerful tool to detect rare high-risk alleles. However, often it is difficult to narrow the region of interest adequately.

Box 1

Types of Genetic Polymorphisms

Polymorphism	**Change**	**Example**
Single-base nucleotide substitutions (also called single nuleotide polymorphim, SNP)	Change of one nucleotide	GR gene: N363S in exon 2, *BclI* in intron 2 [6]
Small-scale multi-base deletions or insertions (also called deletion insertion polymorphism, DIP)	Insertion or deletion of 1-5 nucleotides	ACE gene: intronic 287-base-pair nonsense DNA domain [7]
Microsatellite repeat variations (also called short tandem repeats, STR)	Repeats of a number of nucleotides (2, 3, or 4)	IGF-1 gene: CA repeat in promoter [8] AR gene: CAG repeat in exon 1 [9]

Abbreviations: GR, Glucocorticoid receptor, ACE, angiotensin-converting–enzyme, IGF-1, Insulin-like growth factor 1, AR, androgen receptor.

To detect genes which play a role in common multifactorial diseases with a strong environmental component population-based association studies have become very popular[10, 11].

Association studies test whether a genetic polymorphism occurs more frequently in cases than in healthy controls. Also, certain traits can be studied and compared between carriers and noncarriers of a polymorphism. Most studied polymorphisms are single nucleotide polymorphisms, in which one of the nucleotides is substituted by another one with a frequency within the normal population of more than 1%. Also other types of polymorphisms exist, e.g. microsatellite repeat polymorphisms or deletion/insertion polymorphisms (DIP) (Box 1).
The techniques to detect polymorphisms have rapidly developed in the past few years, especially with the introduction of high-throughput techniques [12]. These facilitated researchers to search for polymorphisms in candidate genes in large numbers of individuals in a simple, cheap and rapid manner. However, many limitations have to be recognized when conducting association studies (Box 2).

Box 2

Fallacies of Polymorphism Studies

* unreliable phenotyping
* low number of individuals studied
* racial heterogeneity
* population stratification (founder effect)
* gender differences
* age differences
* (no) functionality of the studied gene variation
* statistical analysis (false positive results by multiple testing)
* publication bias

An important aspect to assure high quality of an association study is reliable phenotyping. This includes careful recruitment of participants to rule out a bias by incorrect inclusion. As previously discussed by Gambaro et al phenotypic differences between studies can also exist due to variable definitions for cases and controls in different studies, and the heterogeneous phenotypic expression of certain diseases [13]. Sensitivity and specificity of the methods used to characterize phenotype should also be taken into account. This applies to the doctor or researcher who performs measurements, but also for the variance in measurement of routine

parameters such as laboratory measurements. A problem of large-scale population studies, in which many steps of data-collection and many different researchers are involved is the occurrence of (frequently undetected) errors. These errors can occur at many levels, e.g. data-collection, genotyping and information processing. The influence of these errors can be reduced by increasing the sample size, minimizing inter-researcher variation (in e.g. physical examination or evaluation of radiodiagnostics) and building in steps to double-check the measured parameters (Box 3).

Box 3

Requirements to assure high quality of association studies

* good phenotyping (careful recruitment of subjects and data-collection, high sensitivity and specificity of tests, minimizing inter-researcher variation, double-check measured parameters)

* high number of individuals studied (depending on the frequency of the studied gene variant)

* homogeneity of the study population, with respect to ethnicity, gender, age and environmental factors or using statistical corrections for these confounders

* replication in different study population(s)

* a good rationale for the association under investigation to increase the a priori justification

* statistical analysis using multiple testing corrections

* confirming *in vivo* results in *in vitro* experiments

* unraveling the molecular basis of the mechanism of the studied gene variation

A widely discussed topic is the problem of multiple testing. It is recognized that when performing a large number of statistical tests, the rate of false positive results (type I error) is relatively high [14]. One way to diminish this problem is to statistically correct for multiple testing. However, other factors can also help to reduce the amount of false positive results. A good rationale for the association study, creating a high a priori justification, will increase the chance of finding true positive results. With respect to polymorphisms of the glucocorticoid receptor (GR) gene we hypothesized that the sensitivity to glucocorticoids (GCs) is associated with

genetic variants in the GR gene. It is known that GCs exert the majority of their effects through binding to the GR, which makes the GR gene a candidate gene to study the genetic basis of differences in the response to GCs within the normal population. We first performed in vivo studies using dexamethasone suppression tests, in order to investigate whether there were differences in GC sensitivity between carriers and noncarriers of several polymorphisms of the GR: N363S, ER22/23EK (two linked single nucleotide polymorphisms of which the second one results in a amino acid change) and the intronic *Bcl*I polymorphism. For the N363S and the *Bcl*I polymorphisms we found an increased response to the effects of dexamethasone, which indicates an increased sensitivity to the effects of GCs [15, 16]. In contrast, for the ER22/23EK polymorphism we observed a decreased response to dexamethasone, indicating a relative resistance to GCs [17] (see figure 3). These findings gave us a good rationale for our next hypotheses concerning associations of the GR polymorphisms with other parameters affected by GCs, e.g. body composition and metabolic parameters (insulin, lipids). As expected we found associations for the ER22/23EK with measures of decreased GC effects (lower insulin levels, lower cholesterol levels, beneficial body composition, see figure 3)[17, 18], whereas we observed for the N363S and the *Bcl*I associations with measures of increased GC effects (more body fat, higher insulin response to dexamethasone, less lean body mass)[15, 16]. Another way to reduce the rate of false positive results is to increase statistical power by, for example, increasing the sample size [14].

Another important aspect is confirmation of an observed association in a different study population. This diminishes the risk of findings by chance. The phenotypic changes in carriers of the N363S, ER22/23EK and BclI polymorphisms found in the same population wherein the dexamethasone suppression test was performed were in accordance with the observed changes in GC sensitivity. In a study in Dutch elderly healthy individuals we observed that carriers of the BclI polymorphism (G-allele) had a lower body mass index than in noncarriers, which was confirmed in a separate population of elderly Dutch men [16].

However, a risk of replication of an observed association in a different ethnic population is that when the replication fails, the first association can be considered as false positive. It is important to realize that polymorphisms can exert different effects in different ethnicities. First, the frequency of polymorphisms can differ between different ethnic groups. In this respect is it intriguing that for example this N363S genotype, which we found to be 3.1 % (allele frequency) in the elderly healthy Dutch Caucasian population, is highly frequent in an Australian population (allele frequency 7.4 %) [19], whereas in other reports no N363S-carriers have been detected in a Chinese population [20] nor in a Japanese population [21], and an allele frequency of 0.3% in a population of South Asian origin living in northeast England [22].

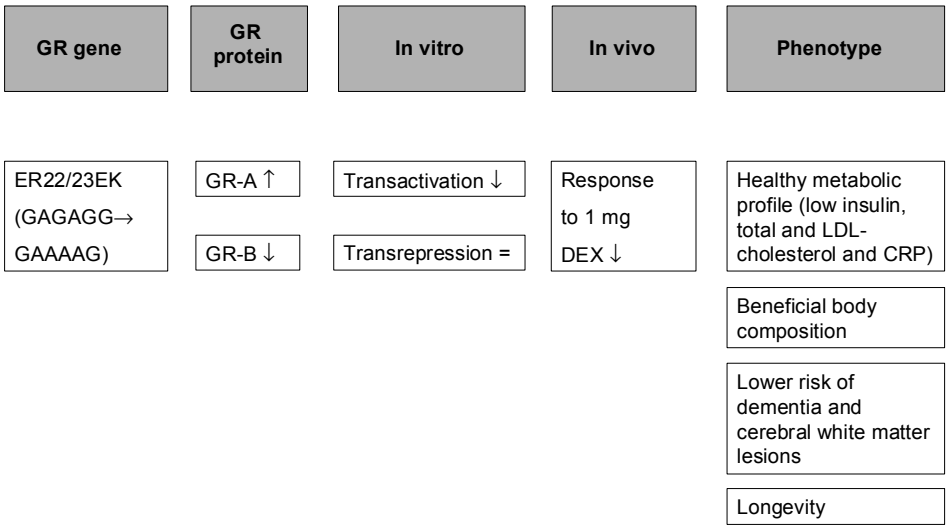


Figure 3: Simplified scheme of a relation between a genetic polymorphism and a phenotype. *The ER22/23EK polymorphism of the glucocorticoid receptor (GR) gene, consists of two single nucleotide polymorphisms in codons 22 and 23, of which the latter results in an amino acid change from arginine (R) to Lysine (K). The ER22/23EK polymorphism probably alters the secondary structure of the mRNA of the GR, forcing a shift towards the usage of Methionine-1 instead of Methionine 27 as startcodon. Due to this alternative usage of Methionine 1 the ER22/23EK polymorphism results in an altered balance [23] of the GR-A (transcriptionally less active) and GR-B (transcriptionally more active) [24] forms at the protein level. In vitro experiments indeed showed a reduced transactivating capacity of the GR-ER22/23EK, whereas transinhibition was unchanged since the different translational isoforms were even potent in inhibiting NF-κB. In addition, the capacity of the GR in the homozygous ER22/23EK carrier to upregulate glucocorticoid-induced leucine zipper (GILZ) protein was less, while transrepression of interleukin-2 (IL-2) did not differ from the control group [25]. After a 1 mg overnight dexamethasone (DEX) suppression test carriers of the ER22/23EK polymorphism had significantly higher cortisol levels than noncarriers, suggesting that ER22/23EK-carriers are relatively resistant to the effects of glucocorticoids with respect to the sensitivity of the negative feedback mechanism[17]. The ER22/23EK polymorphism is associated with a variety of phenotypic changes: better insulin sensitivity, lower total and LDL-cholesterol levels, and lower C-reactive protein (CRP) levels, sex-specific beneficial body composition (greater height, lean mass en muscle strength in male carriers and smaller waist and hip circumferences in female carriers), lower risk on the development of dementia and cerebral white matter lesions, as well as longevity [17, 18, 26, 27]. All these phenotypic changes in ER22/23EK-carriers can be explained by their subtle decreased sensitivity to glucocorticoids.***

Second, the presence of a combination with other polymorphic genes, which is highly variable between ethnicities, can also lead to a different phenotype. Third, environmental factors can contribute to the effects of a certain polymorphism on the phenotype. In our example of the N363S polymorphism of the GR gene, the hypersensitivity to GCs may results in pattern of easier fat storage due to hypersensitive insulin secretion in N363S carriers [15, 28]. In an environment with high fat diet, these carriers are more likely to become obese than in a setting where a low fat diet is more common, and subsequently this variant can result in different phenotypes. In addition, population stratification is a major problem of association studies, which is also referred to as the "founder effect" [10]. This is the tendency that within populations high frequencies of both certain genes as well as certain diseases are present, possibly leading to false positive associations. Lohmueller et al suggested that larger sample sizes, as well as studies with family-based controls can help to avoid this problem and to make results more likely to be replicated [29].

Also sex-specific or age-dependent associations of polymorphisms have been described. An example of a sex-specific association of a polymorphism of the GR gene is the ER22/23EK variant. At young adult age, male ER22/23EK-carriers were on average taller, had more lean mass, and were stronger (see figure 3), whereas female ER22/23EK-carriers showed a tendency towards lower fat mass and smaller waist and hip circumferences [18]. Both associations can be explained by a relative resistance to the effects of GCs, as previously shown in a population including both men and women [17]. However, the reason why different associations with respect to body composition have been found in men and women remains unclear, but hormonal factors may play a role in these differences. An example of an age-dependent association of a GR polymorphism is the relationship of the BclI with body mass index (BMI). In middle-aged subjects this variant has been found to be associated with an increased BMI, as well as waist-to-hip ratio (WHR)[30]. In contrast, in two elderly populations we found this variant to be associated with lower BMI [16]. However, since BMI does not differentiate between fat mass and lean mass we also studied their body composition using DEXA scans. We observed that carriers of the BclI variant allele (G-allele) showed a tendency towards lower lean mass, whereas no differences were found in fat mass, which suggests that these carriers suffered more from sarcopenia during the normal aging process than noncarriers of this variant [16]. Both the increased BMI and WHR in middle-aged individuals (more abdominal fat mass) and the decreased BMI in older persons (more loss of lean mass during aging) can be explained by an increased sensitivity to GCs, as previously shown [16]. In this way polymorphisms can be associated with a certain hormonal or metabolic condition, which can due to numerous other processes throughout the human body result in different phenotypes at different ages. Therefore, in replication studies it is important that besides ethnicity, factors as gender and age are also taken into account.

Another important feature of association studies is that there should be a biological plausibility for a candidate gene. Usually genes are chosen as candidate gene when they code for an important factor in the pathway of a certain process and thus can be involved in the pathophysiology of a disease. Also linkage analysis can lead to the detection of certain candidate genes. After an association between a polymorphism and a phenotype is found, it does not necessarily mean that there is a causal relationship. A polymorphism can be in linkage disequilibrium with another polymorphism in the same gene or even in an adjacent gene.

In vitro testing of the effects of a polymorphism can help to distinguish between functional and non-functional polymorphisms. In our example of the GR gene Russcher et al observed that the ER22/23EK polymorphism influences the transactivating capacity of the glucocorticoid receptor, while transrepression of NF-κB activity is not affected [25]. In addition, Russcher et al studied the upregulatory effects by investigating the effects of the ER22/23EK variant on glucocorticoid-induced leucine zipper (GILZ) protein, as well as the downregulatory effects on interleukin-2 (IL-2). The capacity of the GR in the homozygous ER22/23EK carrier to upregulate GILZ was less, while transrepression of IL-2 was equal to the control group (see figure 3). This was consistent with our findings in vivo of a reduced sensitivity in ER22/23EK-carriers. Russcher et al also studied the in vitro effects of the N363S variant and found that this polymorphism increases the transactivating capacity, both, in vitro and ex vivo [25], which is consistent of our findings of an association of the N363S variant with increased glucocorticoid sensitivity in vivo [15].

Furthermore, the location of the polymorphism within the gene is important with respect to functionality. For example, the N363S polymorphism results in a change of asparagine to serine, which creates a potential phosphorylation site and could be relevant for DNA binding by the GR [31, 32]. However, the exact mechanism of the N363S has not been elucidated at present. In contrast, the molecular mechanism through which the ER22/23EK polymorphism reduces GC sensitivity has recently been clarified by Russcher et al [23]. Yudt et al [24] reported that at least two different methionine codons in the GR mRNA are used as initiation codon: AUG-1 and AUG-27, resulting in two translation variants, the 94 kDa GR-A and the 91 kDa GR-B protein, respectively. The shorter GR-B protein had a stronger transactivating effect in transient transfection experiments [24]. The sensitivity in GR(ER22/23EK)-carriers is decreased, because more of the longer, less transcriptionally active GR-A isoform is formed [23] (see figure 3). The polymorphism probably alters the secondary structure of the mRNA of the GR, forcing more translation initiation from AUG-1. The study of Russcher et al indeed shows a reduced transactivating capacity of the GR-ER22/23EK and can be explained by the changed GR-A/GR-B ratio. Transinhibition seems to be unchanged because the different translational isoforms were equally potent in inhibiting NF-κB [25].

Functionality of intronic polymorphisms remains a difficult issue. Intronic polymorphisms are often considered as nonfunctional, because they do not change the coding sequence. However, they can still be involved in the splicing process for example by changing the sequence of so-called intronic splicing silencers or enhancers or other mechanisms important for the expression of the gene [33].

For the majority of the polymorphisms a molecular mechanism has not been found yet. To study the effects of polymorphisms other methods than studying the associations of one single polymorphism with phenotypic data has become popular over the past few years, e.g. haplotype analysis. Haplotypes have been constructed, consisting of alleles containing a number of polymorphisms throughout one gene and in this way the association of phenotypic changes with certain risk alleles could be identified. In our example of the GR gene, Stevens et al constructed a haplotype and found this to be associated with an increased sensitivity to GCs [34]. Interestingly, the three polymorphisms of the GR gene described in this review sofar were found to exclude each other, i.e. they never occur on the same allele [35], which simplifies the analysis of association studies concerning these polymorphisms.

A problem with respect to association studies which is often discussed is the fallacy of publication bias, whereby journals tend to publish rather positive than negative results [36]. However, by a large meta-analysis comprising 301 publications on 25 associations Lohmueller et al convincingly showed that publication bias seems implausible to account for the inconsistency in the reproducibility of association study results [29]. In eleven of these 25 associations the results were very well replicable (which is however still less than 50 %). Lohmueller et al state that underpowered non-significant studies of real associations with modest genetic effects can reasonably account for much of the variability in replication. This meta-analysis shows again the need for well-designed and sufficiently powered replication studies of every positive association between a common polymorphism and a common complex disease.

Worldwide many genetic data became available by the Human Genome Project, and technical and informatic methods have rapidly improved. In the future a shift towards whole-genome association studies may become apparent [37]. However, also for these future whole-genome association analysis careful study design remains number one priority.

Clinical Relevance

The observed associations with altered sensitivity to GCs may contribute to a better understanding of the variations in regulation of the HPA-axis between normal individuals. Previous data suggest that the setpoint of the HPA-axis in humans might be to a large extent genetically determined, since the intra-individual baseline cortisol concentrations are highly reproducable [38]. These GR gene polymorphisms seem to have modifying effects in conditions such as atherosclerosis. It is known that some individuals survive till a high age, although they

have increased cholesterol levels [39], thus they might be protected by a genetic variant such as the ER22/23EK. On the other hand, individuals who carry the N363S or the BclI polymorphism might be more at risk for cardiovascular disease. The N363S variant recently has been found to be associated with coronary artery disease, independent of obesity, as well as with increased total cholesterol and triglyceride concentrations and an elevated total cholesterol/HDL ratio [40].
In clinical practice, GCs are widely used for the treatment of numerous diseases, such as asthma, chronic inflammations, prevention of rejection of organ transplants, as well as replacement therapy. It is well known that the effects of treatment with GCs vary considerably between patients. Some patients respond very well to the therapeutical administration of GCs, but also develop serious side effects, while others need a very high dose to establish any clinical effect and do suffer less from side effects. The response to GCs of the majority of patients, however, lies between such extremes . It is likely that these polymorphisms are to some extent responsible for the variability in the responses to therapeutically used GCs. In the future, after appropriate additional research, it might be useful to screen for the presence of these GR gene variants to determine an individual's dose of GCs. This dose should be adjusted, taking into account the genetically determined sensitivity to GCs, to a person's need, in such a way that it is therapeutically effective, but does not cause side effects. At present, we do not know whether the altered sensitivity associated with these polymorphisms differs for various types of clinically used GCs and whether the manner of application (local, systemic) influences the effects of the polymorphisms.

With respect to the ER22/23EK polymorphism we studied this variant at various ages (figure 4). During puberty we observed that male carriers showed tendencies towards greater body height, lean mass and greater muscle strength. These differences between carriers and noncarriers were more pronounced and statistically significant at young adult age. In females, we observed associations with smaller waist and hip circumferences, suggesting less central fatmass. At older age, we observed a healthier metabolic profile (better insulin sensitivity and lower total and LDL-cholesterol levels) in ER22/23EK-carriers. Also, the risk of developing dementia, as well as cerebral white matter lesions was reduced. At very high age we found the C-reactive protein levels to be lower in ER22/23EK-carriers compared to noncarriers, possibly reflecting a beneficial vascular condition, as well as a tendency towards lower cholesterol levels. In these elderly males we also observed a reduced mortality rate after a four year follow-up, suggesting the ER22/23EK to be a longevity polymorphism. Although the associations observed with the ER22/23EK polymorphism differ between the various age groups, all effects can be explained by relative cortisol resistance as a result of this DNA sequence alteration.

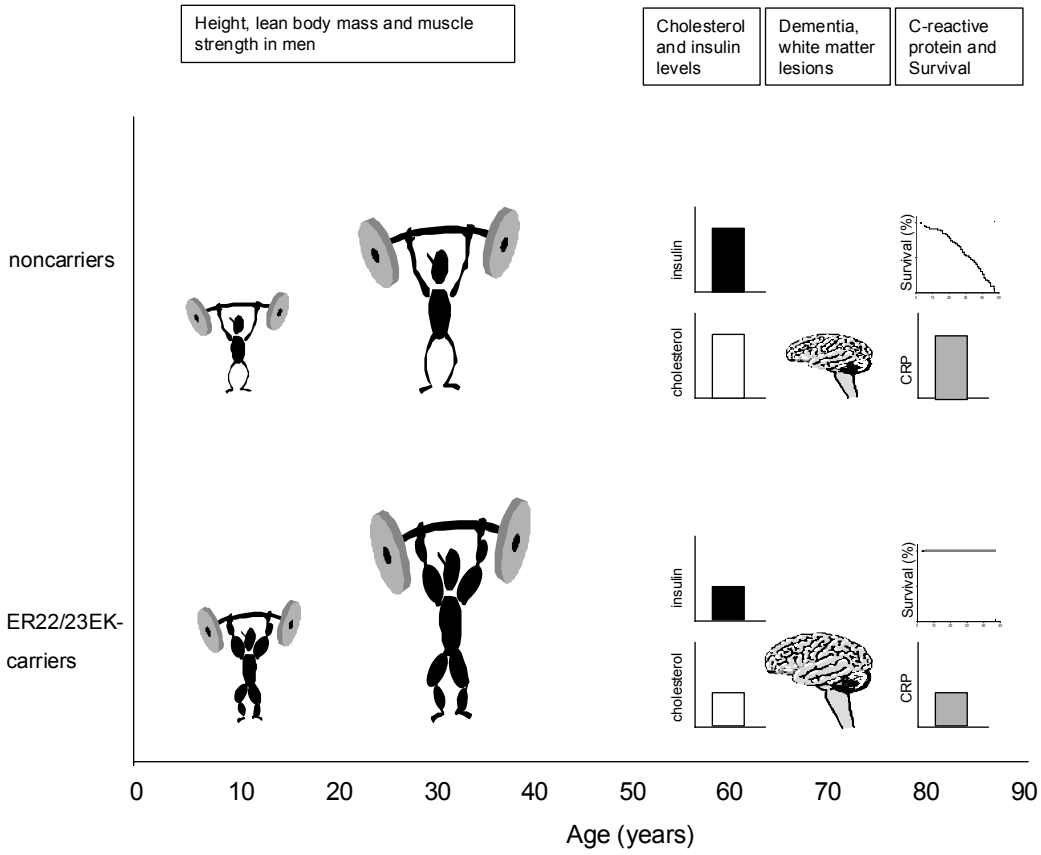


Figure 4: *we studied the ER22/23EK polymorphism at various ages. We observed during puberty that male carriers showed tendencies towards greater body height, lean mass and greater muscle strength. At young adult age these differences between carriers and noncarriers were more pronounced and statistically significant. In females, we observed associations with smaller waist and hip circumferences, suggesting less central fatmass. At older age, we observed a healthy metabolic profile (better insulin sensitivity and lower total and LDL-cholesterol levels). Also the risk of developing dementia, as well as cerebral white matter lesions was reduced. At very high age we found the C-reactive protein levels to be lower in ER22/23EK-carriers compared to noncarriers, possibly reflecting a beneficial vascular condition, as well as a tendency towards lower cholesterol levels. In these elderly males we also observed a reduced mortality rate after a four year follow-up, suggesting the ER22/23EK to be a longevity polymorphism. Although the associations observed with the ER22/23EK polymorphism differ between the various age groups, all effects can be explained by a relative resistance as a result of this DNA sequence alteration.*

As we described in this thesis the ER22/23EK polymorphism has also effects on the brain. The risk of development of dementia and cerebral white matter lesions was reduced in ER22/23EK-carriers. Since cardiovascular risk factors are associated with an increased risk of dementia[10,11] and high cortisol levels have been associated with cognitive impairment and dementia[12-14], these protective effects could be a result of both their decreased sensitivity to GCs as well as their favourable cardiovascular profile.

High cortisol levels have also been associated with depression [41]. In this thesis we described that the *BclI* and ER22/23EK polymorphisms are associated with the susceptibility to develop a major depression. In addtion, the ER22/23EK variant was related to a better response to antidepressant treatment, as well as a slightly better cognitive functioning as tested with the divided attention test during major depression. These findings are in accordance with previous observations that dysregulation of the HPA-axis plays a major, and possibly causal, role in depression, which might be (partially) genetically determined. Recently, Binder et al reported a genetic factor involved in the regulation of the HPA-axis. They found significant associations of response to antidepressants and the recurrence of depressive episodes with single-nucleotide polymorphisms in FKBP5, a GR-regulating cochaperone of hsp-90 [42]. In addition, these single-nucleotide polymorphisms were associated with increased intracellular FKBP5 protein expression. This causes alterations in the GR and thereby these polymorphisms are involved in HPA-axis regulation. Carriers of this specific FKBP5 haplotype had less HPA-axis hyperactivity during a depression. These data suggest that the FKBP5 variant-dependent changes in HPA-axis regulation could be related to the faster response to antidepressant drug treatment and the increased recurrence of depressive episodes observed in this subgroup of depressive patients.

In the future, the in this thesis described associations of the ER22/23EK polymorphism with a protective effect on dementia and white matter lesions, as well as a faster response to antidepressant treatment in depressive patients, might offer possibilities to predict in early stages which individuals are at risk for cognitive impairment and enable to design a more individualized plan for treatment of depression.

Body Composition

Both the N363S and the *BclI* polymorphisms may predispose to the development of obesity. However, as is well known in obesity, environmental, dietary and socioeconomic factors, are also important determinants of the phenotype[43]. Furthermore, the distribution of energy expenditure requirements and individual substrate partitioning are known to influence the energy balance depending on the genetic profile[43, 44]. Weight gain is determined by a positive balance between the amount of energy consumed over the energy spent in everyday life [45]. Evidence is accumulating that genetic factors are involved in these processes. It is commonly

observed that several obese members can be found within one family [46]. Also, the correlation for body mass index (BMI) is higher in monozygotic (0.70-0.88) and dizygotic twins (0.15-0.42) compared to parents and children (0.15-0.23) and husband and wife (0.10-0.19)[47, 48]. In addition, studies of dietary intervention in identical twins showed that the differences in the susceptibility to overfeeding or periods of dietary restriction seem to be partially explained by genetic factors [46]. Many genes have been reported to be involved in the processes leading to obesity. In this context, single gene mutations affecting energy intake (leptin (LEP), leptin receptor (LEPR), pro-opiomelanocortin (POMC), melanocortin 4 receptor (MCR4), protein convertase 1(PC1)) or causing a reduction in energy expenditure (Prader Willi syndrome) resulting in an extremely obese phenotype have been described [49]. Besides genes involved in the regulation of appetite (as mentioned above, as well as neuropeptide Y) body weight is also regulated by variations in energy expenditure (uncoupling proteins) and nutrient utilisation, resting metabolic rate and response to physical activity (adrenergic receptors, fatty acid binding protein), as well as individual differences in adipocyte metabolism (peroxisome proliferator activated receptors (PPAR))[46]. Polymorphisms in the β2-adrenergic receptor gene and the LEPR gene have been reported to be associated with weight gain [50]. Also a polymorphism in the PPARγ2 gene, as well as additive effects of variations of the β3-adrenergic receptor and uncoupling protein 1 genes have been shown to affect weight maintenance after weight loss[51,52].

Environmental factors and lifestyle also influence body composition. In particular excessive caloric intake and a sedendary pattern, which both have become increasingly common in the past decades (high caloric meals and snacks, motorized transport, TV viewing, computer work), contribute to weight gain [53]. The interactions between genetics and sedentarism have been evaluated in twinpairs. From these studies can be concluded that the genetic predisposition may modify the effect of physical activity on weight change and a sedentary lifestyle may have an obesity-inducing effect depending on genetic susceptibility [54, 55]. It is well known that interindividual differences in the reaction to diverse dietary interventions or to physical exercise exist. Differences in genetic make-up may underlie these variations. An example of an interaction between genes and lifestyle is the observation that carriers of a polymorphism of the β3-adrenergic receptor gene have an increased risk of developing obesity when they remain sedentary [56].

With respect to the GR polymorphisms described in this thesis, environmental factors could also play a role in the observed relationships between BMI and genotype. For example the effects of these polymorphisms could be influenced by the amount of stress an individual perceives throughout life. A period of (psychological or physical) stress is accompanied with increased cortisol levels. Cortisol exert its effects mainly via the GR. A person carrying the N363S or the *BclI* polymorphism, leading to an increased sensitivity to GCs, might be more prone to suffer from deleterious effects, e.g. abdominal obesity, diabetes, muscle atrophia,

than noncarriers. In this respect, we observed in a severly obese population that N363S-carriers had an even higher BMI than obese noncarriers [28]. This suggests that the N363S polymorphism might result in increased lipogenesis leading to higher BMI, due to an increased insulin response elicited by a relative hypersensitivity to cortisol. In contrast, ER22/23EK-carriers, who are relative resistant to the effect of cortisol might be protected from these adverse consequences. Previously, N363S-carriers [15, 19] and *BclI*-carriers [57, 58] have been found to have an increased BMI. As described in this thesis we observed that elderly carriers of the *BclI* polymorphism had lower BMI, which may be explained by their tendency towards lower lean body mass [16]. Beside gene-environment interactions also gene-gene interactions exist. In this context, we observed (this thesis) that carriers of both the N363S and *BclI* variant had higher cholesterol levels, as well as a tendency towards higher blood pressures, while the risk of carrying only the N363S or the *BclI* was not associated with an unfavourable cardiovascular profile [28].

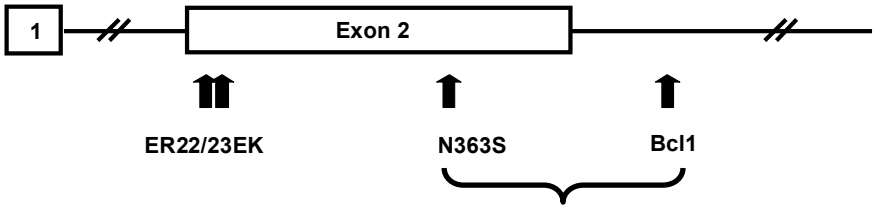
Evolution

During evolution, a selection process occurred in which some de novo mutations probably had beneficial effects and became slowly more frequent in the population. We found that the ER22/23EK variant in males was associated with more lean mass and more muscle strength. In this view, the ER22/23EK polymorphism could have resulted in strong individuals, who had a greater chance to survive due to an advantage in capability of collecting food and defending themselves in fights. The N363S and *BclI* carriers may also have had advantages to survive through their tendency to accumulate fat, which was especially favorable in times of food deficit, or during pregnancy and motherhood to increase survival chances of children. In this respect, the *BclI* polymorphism probably has been arisen long ago, because the allele frequency in the normal population is very high. However, nowadays, in times of abundance of food, in combination with increased psychological stress and a lack of exercise, the N363S and *BclI* polymorphisms may have turned to a disadvantage. An increased sensitivity to GCs, resulting also in fat accumulation is probably one of the risk factors of atherosclerosis. This is supported by the findings of increased risk on coronary artery disease and obesity in N363S-carriers in an Australian population [40, 59].

Conclusion

In conclusion, the N363S, *BclI* and ER22/23EK polymorphisms in the *GR* gene, but not the *TthIIII* polymorphism, are associated with altered GC sensitivity and result in a wide variety of phenotypic signs, which are not pathological per se, but partially explain an individuals genetically determined tendency to a certain body composition, as well as metabolic and mental status (figure 5). The mechanism at a molecular level behind the associations found

with the ER22/23EK variant has been elucidated [23], however to clarify the mechanisms of the N363S and BclI poymorphisms more research is needed.



"Glucocorticoid Resistant"	" Glucocorticoid Hypersensitive"
* Sex-specific better body composition	* More body fat
* Healthier metabolic profile	* Less lean mass
* Longevity	* Hypersensitive insulin secretion
* Lower risk of dementia and cerebral white matter lesions	* Increased cholesterol levels
	* Higher risk on depression
* Higher risk on unipolar recurrent depression and faster response to antidepressant treatment	

Figure 5: *A tentative scheme of the N-terminal part of the glucocorticoid receptor gene, in which three functional polymorphisms are indicated, as well as a summary of their clinical associations.*

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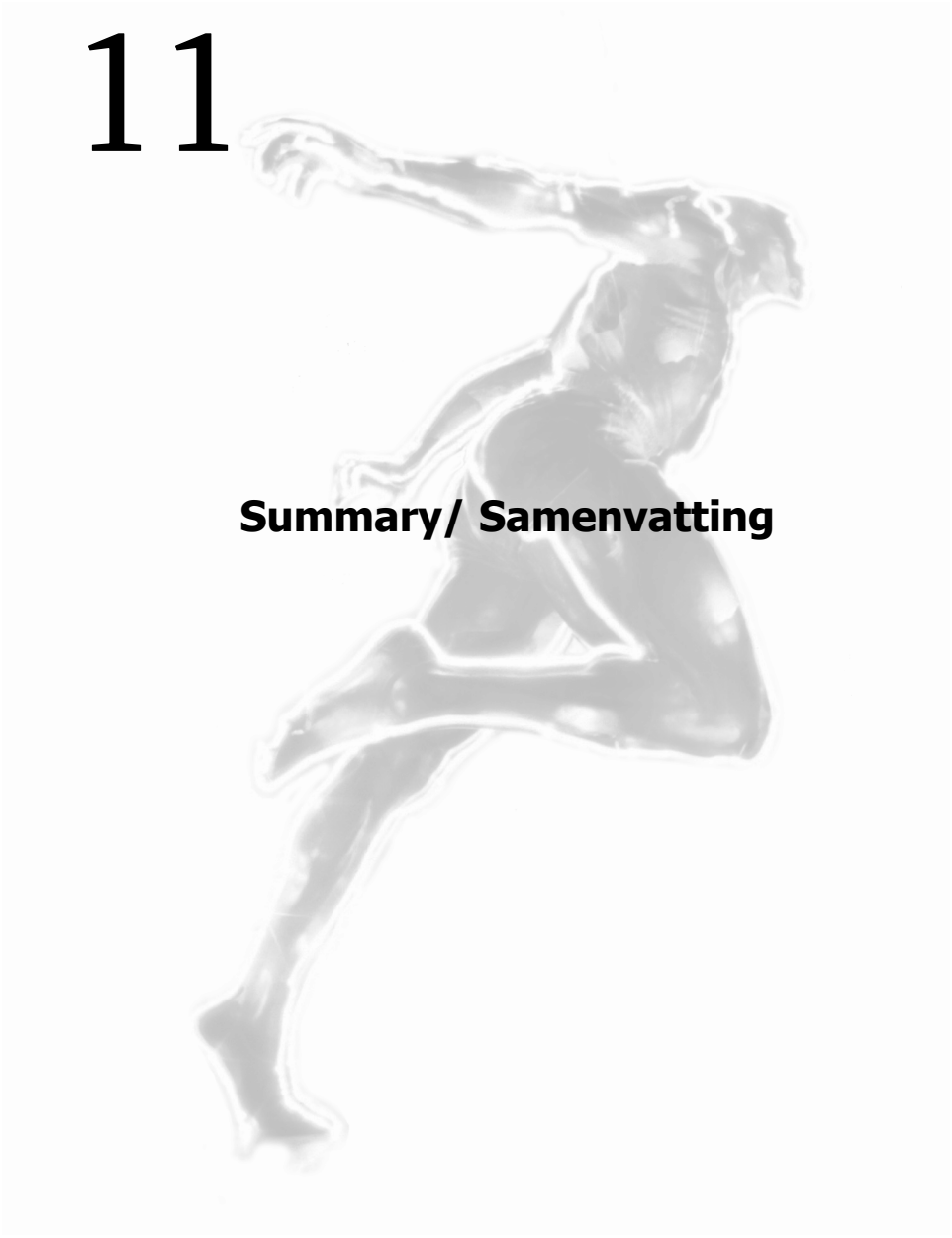

Summary/ Samenvatting

Summary

Glucocorticoids are hormones, of which the production is increased in response to physical or psychological stress. In basal conditions these hormones are also present in the circulation, but in lower levels. The major glucocorticoid in man is cortisol. The effects of glucocorticoids are mainly mediated by the glucocorticoid receptor, which is present in virtually all tissues throughout the body. This thesis is focused on the gene coding for the glucocorticoid receptor.

As introduced in **chapter 1**, it is known that sensitivity to glucocorticoids is highly variable between normal individuals. If glucocorticoids are used therapeutically, the variability of clinical response and adverse effects is considerable as well. In this chapter the background concerning sensitivity to glucocorticoids, several polymorphisms in the glucocorticoid receptor (GR) and the relation to physical and mental status is described. The aim of this thesis was to identify common genetic polymorphisms of the glucocorticoid receptor and to investigate their role of in glucocorticoid sensitivity, metabolism, body composition, dementia and depression.

In **chapter 2** we identified the sequence alteration of an intronic *BclI* restriction site polymorphism of the GR gene as C/G nucleotide change. In 191 healthy elderly, we investigated the relation of this highly frequent variant (allelic frequency 37%) with glucocorticoid sensitivity using a dexamethasone suppression test. Homozygous G allele-carriers, as well as heterozygous G-allele carriers were significantly more sensitive to the suppressive effects of dexamethasone in a allele-dosage way. In a large second study population of healthy elderly we found G-allele carriers to have a lower body mass index (BMI). This was confirmed in a third study population of healthy elderly men. In this latter population we also studied body composition. While fatmass did not differ between genotypes, we found a lower amount of lean mass in homozygous, as well as heterozygous G-allele carriers. This suggests that G-allele carriers suffer more from the loss of lean mass which also occurs during the normal aging process, possibly due to increased glucocorticoid sensitivity.

In **chapter 3** we investigated the role of the previously described N363S polymorphism of the GR gene and the BclI polymorphism in a group of severely obese Italian patients. In this group N363S-carriers had a significantly higher BMI, resting energy expenditure and food intake compared to noncarriers. Carriers of both the N363S and BclI variants had a tendency towards higher systolic and diastolic blood pressures, as well as significantly higher total and LDL-cholesterol levels. Based on these and previous data we speculate that N363S-carriers who turn obese, may easily become even more obese, which might be explained by their hypersensitive insulin response and thus, via activation of lipogenesis, more efficient way of fat storage. In addition, simultaneous carriage of N363S and BclI polymorphisms, both associated with increased glucocorticoid sensitivity, appears to result in a subtle unfavorable cardiovasular risk profile.

In **chapter 4** we identified a TthIII1 restriction site polymorphism, located in the promotor region of the GR gene, as a C/T nucleotide change. This highly frequent polymorphism (allelic frequency 31%) was not related to gluocorticoid sensitivity, as tested with a dexamethasone suppression test in 209 healthy elderly. We also found no correlations between TthIII1 genotype and metabolic parameters or body composition. However, this variant appeared to be linked to the ER22/23EK polymorphism. Carriers of both TthIII1 and ER22/23EK variant alleles were significantly more resistant to the suppressive effects of dexamethasone, and had lower fasting insulin and cholesterol levels compared to carriers of only the TthIII1 polymorphism or noncarriers. Thus, carriage of both TthIII1 and ER22/23EK polymorphisms was associated with a relative resistance to glucocorticoids, and a healthy metabolic profile.

In **chapter 5** we report an association between the ER22/23EK polymorphism of the GR gene and a reduced sensitivity to glucocorticoids, which was studied using a dexamethasone suppression test in 202 healthy elderly. In addition, carriers of the ER22/23EK variant (8.9 %) had lower fasting insulin levels, as well as lower total and LDL-cholesterol concentrations. We also found a significantly higher frequency of the ER22/23EK genotype in the older half of this population compared to the younger half. This is in line with our findings of a relation of the ER22/23EK polymorphism with relative glucocorticoid resistance, resulting in a better metabolic condition.

In **chapter 6** we describe the role of the ER22/23EK polymorphism in body composition. It is known that body composition is closely related to metabolism Considering the findings of the previous chapter we hypothesized that the ER22/23EK polymorphism is also related to the regulation of body composition. Therefore, we investigated a cohort, which was followed from the age of 13 till 36 years. In young adult males we found ER22/23EK-carriers to be taller, have more lean mass, and greater thigh circumferences (indicator of muscle mass), as well as increased muscle strength when compared to noncarriers. This phenotype was already present during puberty, however marked differences could only be detected at adult age. In females, waist and hip cicumferences tended to be smaller, suggestive of less fatmass, but no differences in BMI were found. Thus, the ER22/23EK polymorphism seems to be related to a sex-specific, beneficial body composition at young adult age.

In **chapter 7** we investigated whether the ER22/23EK variant is associated with longevity or predictors of mortality. Therefore, we studied C-reactive protein (CRP) and interleukin-6 (IL-6) levels, as well as cholesterol levels and mortality in 402 men with a mean age of 78 years. After a follow-up of 4 years almost 20 % of the noncarriers died, while none of the 21 ER22/23EK-carriers had died. CRP levels were significantly lower in ER22/23EK-carriers, while IL-6 levels were not related to genotype. CRP levels positively correlated to BMI, total fatmass, and trunk fatmass. Total and LDL-cholesterol levels tended to be lower, but not significantly, in ER22/23EK-carriers. Thus, the ER22/23EK polymorphism is associated with

longevity, as well as with lower CRP levels, which has been shown to be an independent predictor of cardiovascular mortality.

In **chapter 8** we studied the effects of the ER22/23EK polymorphism on the brain. Glucocorticoids are essential for proper brain functioning and increased levels of cortisol have been associated with cognitive impairment and dementia. Therefore, we hypothesized that carriers of the ER22/23EK variant might be protected from the lifelong harmful effects of glucocorticoids on the brain, due to their relative glucocorticoid resistance. In 6034 elderly from the Rotterdam Study we found indeed the ER22/23EK variant to be negatively associated with the risk of developing dementia. In addition, in 1011 elderly of the Rotterdam Scan Study we found that the presence of cerebral white matter lesions and brain infarctions as well as the risk of progression of white matter lesions was decreased in ER22/23EK-carriers. No association was found with atrophy of the medial temporal lobe on MRI. Among non-demented participants, ER22/23EK-carriers had a better performance on psychomotor speed tests than non-carriers, but no differences were found in memory function between genotypes.These results suggest a protective effect of the ER22/23EK polymorphism on the risk of dementia and cerebral small vessel disease.

In **chapter 9** we describe the relationship of three GR polymorphisms (ER22/23EK. N363S, BclI) with major depression. In depressive patients hyperactivity of the hypothalamic-pituitary-adrenal (HPA)-axis is a very well known phenomenon, which may play a role in the pathophysiology of depression. This dysregulation is related to an impaired negative feedback regulation of the glucocorticoid receptor (GR). Because of their inherent differences in glucocorticoid function, carriers of the ER22/23EK, N363S and *BclI* polymorphisms of the GR could be expected to be more or less prone to develop a major depression. Therefore, we studied 496 depressive inpatients and 496 healthy controls. The frequency of the homozygous *BclI* G allele was higher in depressed patients, and the ER22/23EK allele was also more frequent in unipolar, recurrent depressed patients both compared to noncarriers. In addition, ER22/23EK-carriers showed a significantly faster clinical response to antidepressant therapy, as well as a trend towards better cognitive functioning during depression. Thus, the *BclI* GG and ER22/23EK genotypes were associated with susceptibility to develop major depression and the ER22/23EK variant was also related to a faster response to treatment.

Chapter 10 contains a general discussion in which the findings described in this thesis are put into a broader perspective. The fallacies of conducting association studies are discussed, as well as the clinical relevance of our observations, factors that in general determine body composition and aspects of evolution. Finally, we conclude that three polymorphisms in the *GR* gene are associated with altered GC sensitivity and result in a wide variety of phenotypic signs, which partially explain an individuals genetically determined tendency to a certain body composition, as well as metabolic and mental status.

Samenvatting

Als reactie op lichamelijke of psychische stress worden in het menselijk lichaam stresshormonen, zg glucocorticoïden, door de bijnier aangemaakt. In normale omstandigheden zijn deze hormonen ook aanwezig in de circulatie, echter in lagere concentraties. Het belangrijkste glucocorticoïd voor de mens is cortisol. De effecten van glucocorticoïden worden hoofdzakelijk gemedieerd door de glucocorticoïd receptor, welke in bijna alle weefsels in het lichaam aanwezig is. Dit proefschrift gaat over het gen dat codeert voor de glucocorticoïd receptor (GR).

Zoals beschreven in **hoofdstuk 1** is het bekend dat de gevoeligheid voor glucocorticoïden aanzienlijk varieert tussen normale individuen. Wanneer glucocorticoïden therapeutisch worden toegepast is er eveneens sprake van een enorme variabiliteit in de klinische respons en mate van bijwerkingen. In dit hoofdstuk worden de achtergronden met betrekking tot gevoeligheid voor glucocorticoïden, enkele polymorfismen in de GR en de relatie van deze natuurlijke genetische varianten met lichamelijke en mentale condities beschreven.
Het doel van dit proefschrift was om frequent voorkomende genetische polymorfismen van de glucocorticoïd receptor te identificeren en om inzicht te verkrijgen in hun rol in glucocorticoïd gevoeligheid, metabolisme, lichaamssamenstelling, dementie en depressie.

In **hoofdstuk 2** hebben we de mutatie van het *BclI* polymorfisme van het GR gen geïdentificeerd als zijnde een C/G nucleotide verandering. In een groep van 191 gezonde ouderen hebben we de relatie van deze zeer frequent voorkomende variant (allel frequentie 37%) met glucocorticoïd gevoeligheid onderzocht met een dexamethason suppressie test. Homozygote en heterozygote G allel-dragers bleken gevoeliger (dosis-allel effect) voor de suppressieve effecten van dexamethason. In een tweede grote studiepopulatie vonden we dat deze G-allel dragers een lagere body mass index (BMI) hadden. Dit werd bevestigd in een derde studiepopulatie met gezonde oudere mannen. In deze laatste populatie hebben we tevens lichaamssamenstelling onderzocht. De hoeveelheid vetmassa verschilde niet tussen de verschillende genotypes, maar de hoeveelheid vetvrije massa (m.n. spiermassa) daarentegen was lager in zowel homozygote als heterozygote G-allel dragers. Dit suggereert dat G-allel dragers, mogelijk ten gevolge van een verhoogde glucocorticoïd gevoeligheid, op oudere leeftijd extra veel spiermassa verliezen, hetgeen normaliter in enige mate ook al optreedt tijdens het verouderingsproces.

In **hoofdstuk 3** hebben we de rol van het eerder beschreven N363S polymorfisme van het GR gen en het *BclI* polymorfisme onderzocht in een groep Italiaanse patiënten met morbide obesitas. In deze groep bleken N363S-dragers een significant hogere BMI, hoger rustmetabolisme en grotere voedselinname te hebben vergeleken met niet-dragers. Dragers van zowel het N363S als het *BclI* polymorfisme neigden naar hogere systolische en diastolische bloeddrukken, alsmede significant hogere totaal en LDL-cholesterol concentraties in het bloed.

Gezien deze gegevens alsmede eerder beschreven data zou het kunnen dat N363S-dragers die obees worden, mogelijk gemakkelijk nog obeser worden. Dit zou wellicht verklaard kunnen worden door hun hypersensitieve insuline respons en, via activatie van de lipogenese, efficiëntere vetstapeling. Daarnaast blijkt uit deze studie dat het simultane dragerschap van de N363S en *Bcl*I genvarianten, beide geassocieerd met verhoogde glucocorticoïd sensitiviteit, lijkt te resulteren in een iets ongunstiger cardiovasulair risicoprofiel.

In **hoofdstuk 4** hebben we een TthIII1 polymorfisme, gelocaliseerd in de promotor regio van het GR gen, geïdentificeerd als een C/T nucleotide verandering. Dit frequent voorkomende polymorfisme (allel frequentie 31%) was niet gerelateerd aan gluocorticoïd gevoeligheid, hetgeen gemeten werd middels een dexamethason suppressie test in 209 gezonde ouderen. Tevens hebben we geen correlaties gevonden tussen TthIII1 genotype en metabole parameters of lichaamssamenstelling. Deze variant bleek echter partieel gekoppeld aan het ER22/23EK polymorfisme. Dragers van zowel de TthIII1 als ER22/23EK variante allelen waren significant resistenter voor de suppressieve effecten van dexamethason, en hadden lagere nuchtere insuline en cholesterol concentraties vergeleken met dragers van alleen het TthIII1 polymorfisme of niet-dragers. Geconcludeerd kan worden dat dragerschap van beide polymofismen (zowel TthIII1 als ER22/23EK) geassocieerd is met een relatieve resistentie voor glucocorticoïden en een gezond metabool profiel.

In **hoofdstuk 5** beschrijven we een associatie tussen het ER22/23EK polymorfisme van het GR gen en een verminderde gevoeligheid voor glucocorticoïden, hetgeen getest is middels een dexamethason suppressie test in 202 gezonde ouderen. Daarnaast bleek dat dragers van de ER22/23EK variant (8.9 %) lagere insuline spiegels en lagere totaal en LDL-cholesterol concentraties hadden. Ook vonden we een significant hogere frequentie van het ER22/23EK genotype in de oudste helft van deze populatie vergeleken met de jongste helft. Dit komt overeen met onze bevindingen van een relatie van het ER22/23EK polymorfisme met een relatieve glucocorticoïd resistentie, hetgeen resulteert in een betere metabole conditie.

In **hoofdstuk 6** beschrijven we de rol die het ER22/23EK polymorfisme speelt in lichaamssamenstelling. Het is bekend dat lichaamssamenstelling nauw gerelateerd is aan metabolisme. Gezien de bevindingen in het voorgaande hoofdstuk was onze hypothese dat het ER22/23EK polymorfisme tevens gerelateerd is aan de regulatie van lichaamssamenstelling. Om dit te bestuderen hebben we een cohort onderzocht, dat gevolgd en getest werd van de leeftijd van 13 jaar tot 36 jaar. In jong volwassen mannen vonden we dat ER22/23EK-dragers gemiddeld langer zijn, meer spiermassa en een grotere dijbeenomtrek (ook een indicator van hoeveelheid spiermassa) hebben. Daarnaast bleken deze mannelijke dragers ook sterker te zijn dan niet-dragers. Dit fenotype was al gedurende de puberteit in enige mate waarneembaar, echter duidelijke verschillen traden pas op volwassen leeftijd op. In vrouwelijke ER22/23EK-dragers vonden we een neiging tot kleinere heup- en tailleomtrek, hetgeen suggestief is voor minder vetmassa, echter vonden we geen verschillen in BMI. Concluderend is het ER22/23EK

polymorfisme geassocieerd met een sexe-specifieke, gunstige lichaamssamenstelling op jong volwassen leeftijd.

In **hoofdstuk 7** hebben we onderzocht of de ER22/23EK variant geassocieerd is met overleving en voorspellers van mortaliteit. In dit kader hebben we de C-reactive protein (CRP) en interleukin-6 (IL-6) concentraties, evenals cholesterol concentraties en mortaliteit in 402 mannen met een gemiddelde leeftijd van 78 jaar onderzocht. Na een periode van 4 jaar was ca. 20 % van de niet-dragers overleden, terwijl geen enkele van de 21 ER22/23EK-dragers was overleden. De CRP concentraties waren significant lager in ER22/23EK-dragers, terwijl de IL-6 concentraties niet verschilde per genotype. De CRP concentraties waren ook positief gecorreleerd met BMI, de totale hoeveelheid vetmassa en rompvetmassa. Totale and LDL-cholesterol concentraties waren enigszins, maar niet significant, lager in ER22/23EK-dragers. Geconcludeerd kan worden dat het ER22/23EK polymorfisme geassocieerd is met overleving, alsmede met een lagere CRP concentratie, waarvan eerder gebleken is dat het een onafhankelijke voorspeller is van cardiovasculaire mortaliteit.

In **hoofdstuk 8** hebben we de effecten van het ER22/23EK polymorfisme op de hersenen bestudeerd. Glucocorticoïden zijn essentieel voor het goed functioneren van het brein. Verhoogde cortisol concentraties zijn gerelateerd aan cognitieve stoornissen en dementie. Onze hypothese was dat dragers van de ER22/23EK genvariant enigszins beschermd zouden zijn voor de levenslange schadelijke effecten van glucocorticoïden op de hersenen, vanwege hun relatieve glucocorticoïd resistentie. In 6034 ouderen van de Rotterdam Studie onderzochten we of het ER22/23EK polymorfisme geassocieerd is met dementie. Daarnaast bestudeerden we in 1011 ouderen van de Rotterdam Scan Studie de relatie van dit polymorfisme met structurele hersenafwijkingen op MRI. Conform onze hypothese vonden we dat de ER22/23EK variant negatief geassocieerd was met het risico om dementie te ontwikkelen. Daarnaast bleken witte stofafwijkingen en herseninfarcten minder frequent voor te komen in ER22/23EK-dragers. Ook het risico op progressie van witte stofafwijkingen was verlaagd bij dit genotype. We vonden geen relatie met atrofie van de mediale temporaalkwab op MRI. Bij niet-demente deelnemers vonden we dat ER22/23EK-dragers beter scoorden op psychomotore snelheidstesten dan niet-dragers. Er waren echter geen verschillen in geheugenfunctie tussen de genotypes. Deze resultaten suggereren een beschermend effect van het ER22/23EK polymorfisme op het risico op cerebrovasculaire ziekte en met name op dementie.

In **hoofdstuk 9** beschrijven we de relatie van drie GR polymorfismen (ER22/23EK, N363S, BclI) en depressie. In depressieve patiënten is hyperactiviteit van de hypothalamus-hypofyse-bijnier as een bekend fenomeen, wat mogelijk een rol speelt in de pathofysiologie van depressie. Deze dysregulatie is gerelateerd aan een verstoorde regulatie van de negatieve terugkoppeling (feedback) van de GR. Vanwege hun verschillen in glucocorticoïd effect zouden dragers van de ER22/23EK, N363S en *BclI* polymorfismen van de GR meer of juist minder risico

kunnen hebben om een ernstige depressie te ontwikkelen. Om dit te onderzoeken hebben we 496 depressieve patiënten en 496 gezonde controles onderzocht. De frequentie van het homozygote *Bcl*I G allel was hoger in depressieve patiënten dan in controles. Het ER22/23EK allel kwam ook frequenter voor bij unipolaire, recidiverend depressieve patiënten. Daarnaast was er bij ER22/23EK-dragers sprake van een significant snellere klinische respons op antidepressieve therapie, evenals een tendens tot een beter cognitief functioneren gedurende een depressieve episode. Hieruit kan geconcludeerd worden dat de *Bcl*I GG en ER22/23EK genotypes geassocieerd zijn met een verhoogd risico op het ontwikkelen van een ernstige depressie. De ER22/23EK variant bleek tevens gerelateerd aan een snellere respons op behandeling.

Hoofdstuk 10 bevat een algemene discussie, waarin de bevindingen beschreven in dit proefschrift in een bredere context worden geplaatst. De valkuilen van het uitvoeren van associatie studies worden besproken, evenals de klinische relevantie van onze bevindingen, factoren die in het algemeen bijdragen aan de regulatie van lichaamssamenstelling en enkele aspecten van de evolutie. Tot slot wordt geconcludeerd dat drie polymofismen van het GR gen geassocieerd zijn met veranderde glucocorticoïd gevoeligheid en resulteren in een scala van fenotypische symptomen, die gedeeltelijk de individuele, genetisch bepaalde neiging tot een bepaalde lichaamssamenstelling en metabole en mentale conditie kunnen verklaren.

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Erkenningen

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