

# A Polymorphism in the Glucocorticoid Receptor Gene May Be Associated with an Increased Sensitivity to Glucocorticoids *in Vivo*\*

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## ABSTRACT

We investigated whether a polymorphism at nucleotide position 1220, resulting in an asparagine-to-serine change at codon 363 in the glucocorticoid receptor (GR) gene is associated with an altered sensitivity to glucocorticoids.

In a group of 216 elderly persons, 13 heterozygotes for the N363S polymorphism were identified by PCR/single strand conformation polymorphism analysis. In 2 dexamethasone (DEX) suppression tests (DSTs), using 1 and 0.25 mg DEX, the circulating cortisol and insulin concentrations were compared between N363S carriers and controls. In the 1-mg DST, there were no differences between N363S carriers and controls, with respect to adrenal suppression, but there was a significantly higher ( $P < 0.05$ ) insulin response in N363S carriers. In the 0.25-mg DST, a significantly larger ( $P < 0.05$ ) cortisol suppression and higher ( $P < 0.05$ ) insulin response were seen in N363S carriers. Comparison of blood pressure, body mass index (BMI), and bone mineral density (BMD) between the N363S carriers and controls showed that N363S carriers had a higher ( $P < 0.05$ ) BMI but normal blood pressure. There was an obvious trend towards lower age-, BMI-,

and sex-adjusted BMD in the lumbar spine in N363S carriers. GR characteristics measured in 41 controls and 9 N363S carriers in peripheral mononuclear leucocytes showed no differences between N363S carriers and controls, with respect to GR number and ligand binding affinity. However, there was a trend towards greater sensitivity to DEX in the carriers' lymphocytes, in a mitogen-induced cell proliferation assay. In transfection assays, the capacity of the codon 363 variant to activate mouse mammary tumor virus promoter-mediated transcription in COS-1 cells was unaltered, when compared with the wild-type GR.

We conclude that in 6.0% of our study population, a polymorphism in codon 363 of the GR gene was found. Individuals carrying this polymorphism seemed healthy at clinical examination but had a higher sensitivity to exogenously administered glucocorticoids, with respect to both cortisol suppression and insulin response. Life-long exposure to the mutated allele may be accompanied by an increased BMI and a lowered BMD in the lumbar spine but does not affect blood pressure. (*J Clin Endocrinol Metab* 83: 144–151, 1998)

THERE IS a considerable variability in the sensitivity to glucocorticoids (GCs) across individuals. Early morning serum cortisol concentrations show a great diversity among individuals. On the other hand, there is a marked individual stability of baseline cortisol concentrations within persons that is closely related to the feedback sensitivity of the hypothalamo-pituitary-adrenal-axis to a low dose of dexamethasone (DEX) (Huizenga *et al.* 1997, submitted). Extreme examples of variability in sensitivity to GCs are the cortisol hyperreactive syndrome and cortisol resistance (CR). In 1990, Iida *et al.* (1) described a patient who presented with signs and symptoms of Cushing's syndrome, in spite of

persistent hypocortisolemia. So far, this is the only case reported with cortisol hyperreactivity. CR is a rare condition, as well. The first patient was described in 1976 (2), and since then, only about 10 symptomatic and asymptomatic patients and family members have been identified (3–5). As early as 15 yr ago, it was suggested that CR might be the result of a defect in the functional characteristics of the glucocorticoid receptor (GR). The GR has been cloned and sequenced (6) and found to be organized into a series of discrete domains that mediate the receptor functions of hormone-binding, DNA-binding, and transcriptional modulation (7). The binding of GCs to the GR induces a series of cellular events that results in the activation or repression of a network of GC-responsive genes and produces a specific cellular response (8). Thus far, the molecular basis of CR has been elucidated in 4 kindreds (9–12). In all reported cases, mutations in the hormone-binding domain of the GR-gene seemed to be the cause of CR. The molecular basis of cortisol hyperreactivity (1) has not been elucidated yet.

Variability in the sensitivity to GCs is also observed in patients treated with GCs. GCs are important therapeutic

Received April 28, 1997. Revision received September 11, 1997. Accepted September 30, 1997.

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\* This study was supported by a grant from the Dutch Organization for Scientific Research Nederlandse Organisatie voor Wetenschappelijk Onderzoek.

agents used for the treatment of various inflammatory and autoimmune diseases. Although plasma concentrations of GCs can be ascertained, their functional effects on target tissues are very difficult to predict (13). In clinical observations, a considerable variability among subjects is seen in their sensitivity to GC therapy, both with regard to efficacy and to the prevalence and severity of side effects.

In a previous study, 2 DEX suppression tests (DSTs) were performed in 216 elderly individuals, to gain insight into the sensitivity to GCs in the normal population (Huizenga *et al.* 1997, submitted). Furthermore, a general screening of the GR-gene in 40 persons was performed using PCR/SSCP analysis (14). Based on indications that a previously reported polymorphism in the 3'-region of exon 2 [which encodes for the transactivation domain of the GR of the GR gene (Asn363Ser, N363S, Fig. 1C)] might be associated with an increased sensitivity to GCs (14), we reexamined our study population for this polymorphism and analyzed whether its presence was associated with increased GC-sensitivity.

**Materials and Methods**

For the present study, a sample of participants from the Rotterdam Study was invited for an additional examination. The Rotterdam Study is a population-based cohort study of the determinants of chronic dis-

abling diseases in the elderly. All approximately 10,000 inhabitants of a suburb of Rotterdam, aged 55 yr and over, were invited to participate as described elsewhere (15). The population for the present study included 216 persons aged 55 to 80 yr [102 men and 114 women with mean ages of  $67.7 \pm 5.6$  (SE) and  $65.8 \pm 6.1$  yr, respectively] who had completed the baseline visit for the Rotterdam Study not more than 6 months earlier. Subjects with acute, psychiatric, or endocrine diseases, including diabetes mellitus treated with medication, were not invited. Compared with other participants of the Rotterdam Study of the same age without diabetes mellitus, there were no differences in age and gender distribution and cardiovascular risk factors. From all subjects, written informed consent was obtained, and the study was approved by the Medical Ethics Committee of the Erasmus University Medical School.

Two DSTs were carried out as described previously (Huizenga *et al.*, 1997, submitted). In brief, participants were seen at the research centre after an overnight fast. Blood was drawn by venapuncture to determine serum cortisol and insulin concentrations. Participants were given a tablet of 1.00 mg DEX and instructed to ingest it at 2300 h. Next morning, fasting blood samples were obtained at the same time as the previous morning. In these samples, cortisol, insulin, and DEX concentrations were measured. Circulating cortisol DEX concentrations were determined as described previously (Huizenga *et al.*, 1997, submitted). Serum insulin concentrations were determined using a commercially available RIA (Medgenix Diagnostics, Brussels, Belgium). Intra- and interassay variations were 8.0 and 13.7%, respectively.

Two and a half years later, all 216 participants were invited for a 0.25-mg DST; 164 subjects agreed to participate in this second test (76 men and 88 women, with mean ages of  $69.1 \pm 5.9$  and  $67.6 \pm 5.6$  yr, respectively). The same procedures were used as described for the 1-mg

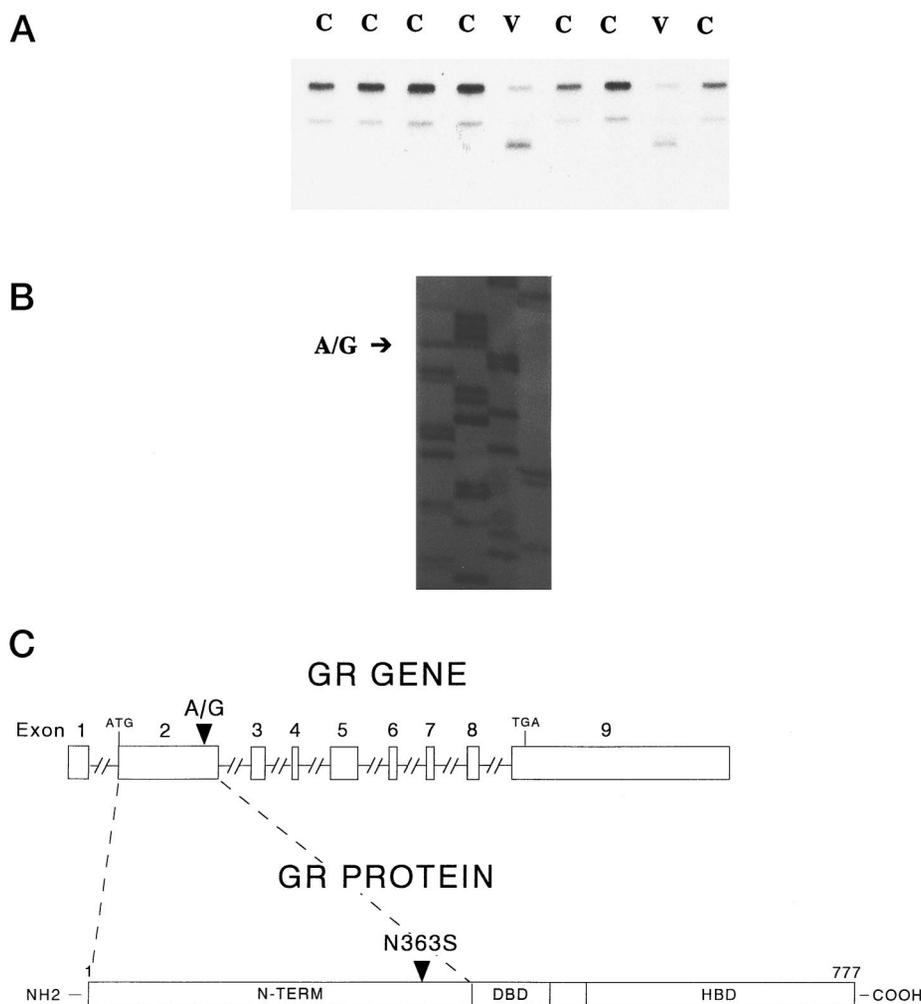


FIG. 1. A, PCR/SSCP analysis of the 3'-part of exon II of the GR gene. The analysis revealed a polymorphism in 13 of 216 elderly individuals (C, control; V, variant). B, Sequence analysis of the polymorphism in the 3'-part of exon II of the GR gene, showing a heterozygous A-to-G point mutation at cDNA position 1220. C, Structure of the human GR gene and protein and its functional domains (DBD, DNA-binding domain; HBD, hormone-binding domain), indicating the position of an asparagine-to-serine change at codon 363, as a result of the A-to-G point mutation at cDNA position 1220 of the GR gene.

DST. In the fasting and post 0.25-mg DEX samples, glucose concentrations were determined using routine standard laboratory methods.

Both as part of the baseline examinations of the Rotterdam Study and at the second determination, body weight and height were measured to calculate body mass index (BMI, kg/m<sup>2</sup>), and sitting blood pressure was measured at the right upper arm with a random-zero sphygmomanometer. Bone mineral density (BMD) measurements were performed by dual-energy x-ray absorptiometry using a DPX-L densitometer (Lunar Radiation Corporation, Madison, WI). 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 prosthesis implantation, the left femur was scanned. Using standard software, the vertebrae L2 to L4 and the femoral neck were analyzed. 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 and 3.2% in the femoral neck (16).

#### PCR/SSCP analysis of the GR gene

DNA was isolated from peripheral blood leucocytes of all subjects using standard techniques. PCR amplification and SSCP analysis of the 3'-region of exon 2 of the GR gene were carried out using primer sequences and amplification and electrophoresis conditions previously described by Koper *et al.* (14).

#### Whole-cell DEX-binding assay and mitogen-induced proliferation assay

From 41 randomly selected controls and 9 carriers of the N363S mutation (see *Results*), 40 mL heparinized blood was drawn for a whole-cell DEX-binding assay and a mitogen-induced proliferation assay. GR characteristics in mononuclear leucocytes and the sensitivity of mononuclear leucocytes to the inhibition of phytohemagglutinin-stimulated incorporation of [<sup>3</sup>H]-thymidine by DEX were determined as described previously (3).

#### Cell culture and transfections

Monkey kidney (COS-1) cells were maintained in DMEM-Ham's F-12 tissue culture medium (Life Technologies, Gaithersburg, MD), supplemented with 5% dextran-coated charcoal-treated FCS (Life Technologies). For transcription regulation studies, cells were plated at  $1.0 \times 10^5$  cells/well (10 cm<sup>2</sup>), grown for 24 h, and transfected overnight by calcium phosphate precipitation, as described previously (17). Cells were transfected with 250 ng GR expression plasmid and 250 ng reporter plasmid per well. After transfection, experimental media were added. After an incubation period of 24 h, cells were harvested for the luciferase (LUC) assay, as described previously (18).

#### Statistical analysis

Results are reported as mean  $\pm$  SE unless otherwise stated. Serum cortisol, insulin and glucose concentrations, blood pressure, BMI, BMD, and receptor characteristics were compared between N363S carriers and controls using Student's *t* test. To control for possible confounders, the analyses were adjusted for BMI, if appropriate, using multiple linear regression analysis. Values for BMD were expressed as Z-scores (number of standard deviations below or above the age-, BMI-, and sex-standardized average value obtained from 2446 males and 3368 females who participated in the baseline visit for the Rotterdam study) (16).

#### Results

After the 1-mg DST, 5 individuals were identified with a post-DEX cortisol concentration >140 nmol/L, which is the cut-off point for a normal test result in our clinic when the DST is used for the screening for Cushing's syndrome. These 5 subjects were further investigated for CR (14) and were left out of the calculations described here. Screening for the codon 363 polymorphism was performed using PCR/SSCP analysis. The abnormal pattern in the 3'-fragment of exon 2 was found in 13 individuals out of the whole study population of 216 subjects (6.0%, Fig. 1A). Sequence analysis of the fragment showed an A-to-G point mutation at complementary DNA (cDNA) position 1220, resulting in an asparagine-to-serine change at codon 363 (Fig. 1B). The mutation was found to be heterozygous in all cases.

The subjects without the mutation in codon 363 (controls) and the 13 persons heterozygous for the polymorphism (N363S carriers) have been investigated further, with respect to their hypothalamo-pituitary-adrenal-axis and sensitivity to GCs.

#### *In vivo* results

The group of N363S carriers consisted of four men and nine women, whereas in the control-group, the sexes were equally divided. There was one female N363S carrier who showed an additional mutation in the 5'-prime part of exon 2 of the GR gene. She was further investigated for CR (14) and left out of all comparisons.

At the first examination, no significant difference in age between the groups was present, as shown in Table 1a. The

**TABLE 1a.** Age, BMI, and blood pressure at first examination in controls (n = 198) and N363S carriers (n = 12)

	Controls		N363S carriers		P-value <sup>a</sup>
	Mean	SE	Mean	SE	
Age (yr)	66.6	0.44	68.5	1.64	0.28
BMI (kg/m <sup>2</sup> )	26.6	0.26	28.1	1.09	0.07
Systolic blood pressure (mmHg)	139	1.35	137	7.00	0.77
Diastolic blood pressure (mmHg)	74.9	0.71	73.1	2.84	0.54

<sup>a</sup> Test for the difference between controls and N363S carriers.

**TABLE 1b.** Age, BMI, and blood pressure at second examination in controls (n = 153) and N363S carriers (n = 8)

	Controls		N363S carriers		P-value <sup>a</sup>
	Mean	SE	Mean	SE	
Age (yr)	68.3	0.49	69.3	2.36	0.63
BMI (kg/m <sup>2</sup> )	25.5	0.30	28.3	1.52	0.04
Systolic blood pressure (mmHg)	143	1.50	140	10.7	0.57
Diastolic blood pressure (mmHg)	88.1	0.86	83.7	6.37	0.26

<sup>a</sup> Test for the difference between controls and N363S carriers.

N363S carriers had a higher mean BMI, compared with controls, which did not reach statistical significance ( $P = 0.07$ ). Systolic and diastolic blood pressures were not different between the groups (Table 1a).

At the second examination, 164 of the initial 216 individuals participated, 9 of whom had the codon 363 polymorphism. The female N363S carrier with the additional point mutation in exon 2 was excluded again from the comparison, and so were 2 subjects who had a post-DEX cortisol concentration more than 140 nmol at the first determination (Table 1b). In the control group, the sexes were equally divided; the group of N363S carriers now consisted of 5 women and 3 men. At the second examination, the N363S carriers had a significantly higher BMI, as compared with the control group ( $P < 0.04$ ). In the first determination, there was no statistically significant difference in BMI between controls and N363S carriers in this subgroup ( $26.1 \pm 0.28$  in controls *vs.*  $28.1 \pm 1.30$  in N363S carriers,  $P = 0.09$ ). As in the first examination, there were no differences between the groups, with respect to systolic or diastolic blood pressure.

Physical examination of the N363S carriers revealed no evident abnormalities. One male N363S carrier had hypertension, and two females had hyperinsulinemia (fasting insulin  $> 25$  mU/L).

**Cortisol concentrations.** Table 2a shows the concentrations of fasting serum cortisol before and after administration of 1 mg DEX, the DEX concentration, and the cortisol suppression in reaction to DEX ( $\Delta$  cortisol). There were no differences between the controls and the N363S carriers, either in basal levels or in response to the administration of 1 mg DEX.

Table 2b provides the same parameters before and after the administration of 0.25 mg DEX. Again, there were no differences in baseline cortisol, but there was a larger decrease in cortisol concentration ( $\Delta$  cortisol) in response to 0.25 mg DEX in the N363S carriers than in controls (280.5 nmol/L in controls *vs.* 373.9 nmol/L in N363S carriers,  $P < 0.09$ , unadjusted data). This difference in  $\Delta$  cortisol was statistically significant when the data were adjusted for BMI (443.8 in controls *vs.* 553.6 in N363S carriers,  $P < 0.05$ ). Adjusting for BMI was necessary because there was a statistically significant difference in BMI between controls and N363S carriers,

and BMI is known to influence the cortisol concentration. The actual DEX concentrations were equal in both groups ( $P = 0.50$ ), so the higher response in the N363S carriers was not caused by higher DEX concentrations.

**Insulin and glucose concentrations.** Table 3 shows the fasting insulin concentrations before and after the administration of 1 and 0.25 mg DEX, respectively, and the increase in insulin levels in response to DEX administration. To be certain that only the data from subjects with a normal carbohydrate tolerance were analyzed, subjects with hyperinsulinemia or diabetes mellitus (fasting insulin or glucose concentrations above 25 mU/L or 7.8 mmol/L, respectively) were excluded from this analysis (20 controls excluded,  $n = 178$ ; and 2 N363S carriers excluded,  $n = 10$ ). There was a significant increase in insulin concentrations in response to the administration of 1 mg DEX among the remaining 188 subjects ( $12.9 \pm 0.53$  mU/L before and  $19.1 \pm 0.80$  mU/L after DEX administration, respectively,  $P < 0.001$ ). There were no significant differences between the groups in fasting or post-DEX insulin concentrations, but there was a significantly larger increase in insulin levels in the N363S carriers in response to 1 mg DEX ( $P < 0.01$ ), compared with controls. As mentioned before, these differences in insulin response were not caused by differences in DEX concentrations between the two groups (Table 2a).

After the administration of 0.25 mg DEX, there was a drop in insulin concentrations in the controls. In the N363S carriers, there was a slight increase in insulin concentrations. After comparison of the insulin responses to 0.25 mg DEX between the two groups, again a significantly larger response in the N363S carriers became apparent ( $P < 0.03$ ). Glucose levels did not increase in response to DEX administration, nor were there differences in glucose concentrations between the two groups.

**BMD.** In Fig. 2, the mean values and SD for BMD in the lumbar spine and the femoral neck in controls and N363S carriers expressed as Z-scores are shown. The figure shows that in the lumbar spine, there was a trend towards lower BMD in the N363S carriers, compared with controls (z-scores:  $-0.48$  *vs.*  $0.02$  respectively,  $P = 0.08$ ). In the femoral neck, there were no differences in BMD between the two groups ( $P = 0.67$ ).

**TABLE 2a.** Cortisol and DEX concentrations in controls ( $n = 198$ ) and N363S carriers ( $n = 12$ ) before and after 1 mg DEX

	Controls		N363S carriers		P-value <sup>a</sup>
	Mean	SE	Mean	SE	
Fasting cortisol (nmol/l)	513.5	11.1	532.2	32.4	0.49
Post DEX cortisol (nmol/L)	26.26	1.22	25.80	3.45	0.97
$\Delta$ cortisol (nmol/L)	488.1	10.9	506.3	33.5	0.50
DEX (nmol/L)	7.300	0.25	8.500	1.44	0.30

<sup>a</sup> Test for the difference between controls and N363S carriers, adjusted for BMI.

**TABLE 2b.** Cortisol and DEX concentrations in controls ( $n = 153$ ) and N363S carriers ( $n = 8$ ) before and after 0.25 mg DEX

	Controls		N363S carriers		P-value <sup>a</sup>
	Mean	SE	Mean	SE	
Fasting cortisol (nmol/L)	541.7	12.0	566.5	44.9	0.53
Post DEX cortisol (nmol/L)	261.2	11.4	192.6	38.1	0.13
$\Delta$ cortisol (nmol/L)	280.5	12.6	373.9	37.2	<0.05
DEX (nmol/L)	2.820	0.13	3.300	0.55	0.48

<sup>a</sup> Test for the difference between controls and N363S carriers, adjusted for BMI.

**TABLE 3a.** Insulin concentrations in controls (n = 178) and N363S carriers (n = 10) before and after 1 mg of DEX

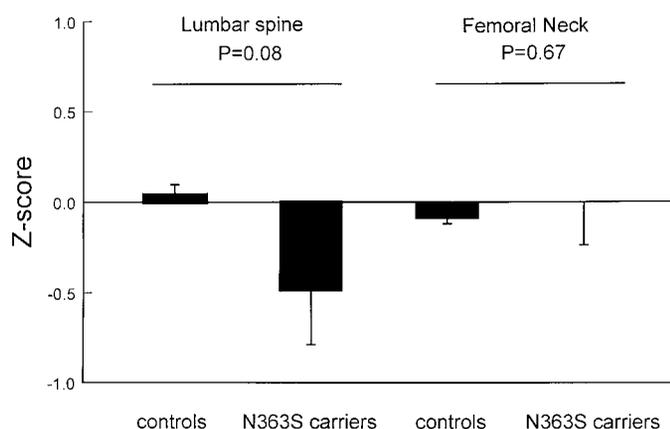
	Controls		N363S carriers		P-value <sup>a</sup>
	Mean	SE	Mean	SE	
Fasting insulin (mU/L)	11.8	0.39	11.2	1.69	0.34
Post DEX insulin (mU/L)	17.6	0.67	23.5	5.19	0.08
Δ insulin (mU/L)	5.84	0.57	12.3	4.09	<0.01

<sup>a</sup> Test for the difference between controls and N363S carriers, adjusted for BMI. Subjects with fasting insulin more than 25 mU/L were left out of the calculation; 2 N363S carriers and 20 controls were excluded.

**TABLE 3b.** Insulin concentrations in controls (n = 127) and N363S carriers (n = 7) before and after 0.25 mg of DEX

	Controls		N363S carriers		P-value <sup>a</sup>
	Mean	SE	Mean	SE	
Fasting insulin (mU/L)	14.8	0.46	13.7	2.29	0.16
Post DEX insulin (mU/L)	13.7	0.51	15.5	2.83	0.69
Δ insulin (mU/L)	-1.10	0.36	1.80	1.98	0.03
Fasting glucose (mmol/L)	5.60	0.06	5.40	0.29	0.26
Post DEX glucose (mmol/L)	5.48	0.05	5.50	0.25	0.75
Δ glucose (mmol/L)	-0.12	0.04	0.07	0.09	0.19

<sup>a</sup> Test for the difference between controls and N363S carriers, adjusted for BMI. Subjects with fasting insulin more than 25 mU/L or fasting glucose more than 7.8 mmol/L were left out of the calculation; 1 N363S carrier and 26 controls were excluded.



**FIG. 2.** Mean values and SD for BMD in the lumbar spine and the femoral neck in controls and N363S carriers, expressed as Z-scores. There is a trend towards lower BMD in the lumbar spine in N363S carriers, compared with controls (Z-scores: -0.48 in N363S carriers vs. 0.02 in controls,  $P = 0.08$ ). There are no differences in femoral neck BMD between the two groups ( $P = 0.67$ ).

### *In vitro* results

#### Whole-cell DEX-binding assay and mitogen-induced proliferation assay

Figure 3 shows the results from 50 whole-cell DEX-binding assays and mitogen-induced proliferation assays (41 randomly selected controls and 9 N363S carriers). The number of receptors (n, Fig. 3A) and the dissociation constant ( $K_d$ , Fig. 3B) were not different in both groups (n = 7056 ± 200 in controls, n = 7242 ± 645 in N363S carriers;  $K_d = 10.6 ± 0.63$  in controls,  $K_d = 8.5 ± 1.14$  in N363S carriers, respectively). Also, no statistically significant difference in 50% of the maximal inhibition ( $IC_{50}$ ) values between the N363S carriers and controls was found; as shown in Fig. 3C, there was a trend towards a lower  $IC_{50}$  in the N363S carriers ( $IC_{50} = 47.7 ± 9.6$  in controls,  $IC_{50} = 21.8 ± 6.4$  in N363S carriers, respectively).

**Transcriptional activation studies.** To investigate whether the observed codon change at position 363 resulted in an altered capacity of the GR protein to activate transcription, we co-transfected a plasmid containing this variant receptor and a mouse mammary tumor virus (MMTV)-driven LUC reporter plasmid in COS-1 cells. Cells were incubated with increasing concentrations of DEX. At DEX concentrations between 0 and 100 nmol/L, the capacity of the N363S variant to activate transcription from the MMTV promoter of the N363S variant was unaltered, compared with the wild-type GR (Fig. 4).

### Discussion

In this study, we examined differences in the sensitivity to GCs within a normal elderly population, using DSTs with two different doses of DEX. In a previous study (14), looking for mutations in the GR gene that cause the clinical syndrome of CR (9–12), we found a polymorphism that seemed to be associated with increased sensitivity to GCs. This polymorphism, located at nucleotide position 1220 (AAT to AGT), results in an asparagine-to-serine change in codon 363 of the GR protein. This polymorphism was heterozygously present in 13 of the 216 subjects. One of these subjects had hypertension, and 2 had elevated insulin concentrations. The other N363S carriers were healthy elderly individuals and showed no signs or symptoms of CR.

A 1-mg DST, carried out to assess differences in GC sensitivity, showed no differences in early morning post-DEX cortisol concentrations. Because 1 mg may be a relatively high dose (93% of the subjects suppressed to a level ≤50 nmol/L), we also carried out a 0.25-mg DST. In this experiment, post-DEX cortisol concentrations showed a much broader range than after 1 mg DEX (Huizenga *et al.*, 1997, submitted). The decrease in early morning cortisol levels (Δ cortisol) was significantly greater in N363S carriers than in controls. The absolute post-DEX cortisol levels also were lower in N363S carriers, but this difference was not statistically significant.

Another parameter for the sensitivity to exogenously administered GCs is the fasting insulin concentration before

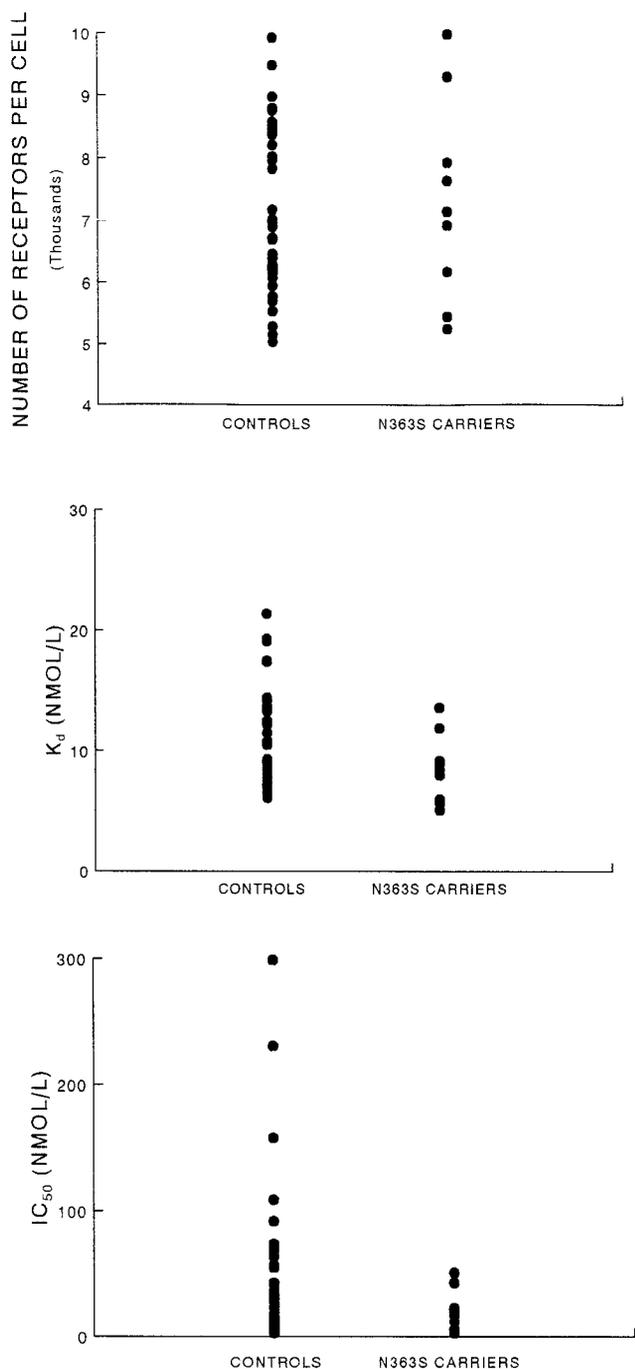


FIG. 3. GR characteristics in peripheral blood mononuclear leucocytes in 41 randomly selected controls and 9 N363S carriers. A, Number of receptors in controls and N363S carriers obtained from whole-cell DEX-binding assays. There is no difference between controls and N363S carriers in number of receptors per cell ( $n = 7056 \pm 200$  in controls;  $n = 7242 \pm 645$  in N363S carriers,  $P < 0.72$ ). B, Ligand affinity of the receptors ( $K_d$ ) in controls and N363S carriers obtained from whole-cell DEX-binding assays. There is no difference in  $K_d$  between controls and N363S carriers ( $K_d = 10.6 \pm 0.63$  in controls;  $K_d = 8.5 \pm 1.14$  in N363S carriers,  $P < 0.17$ ). C, Concentrations of DEX necessary to achieve  $IC_{50}$  in mitogen-induced proliferation assays in controls and N363S carriers. There are no differences in  $IC_{50}$  values between controls and N363S carriers ( $IC_{50} = 47.7 \pm 9.6$  in controls;  $IC_{50} = 21.8 \pm 6.4$  in N363S carriers,  $P < 0.23$ ).

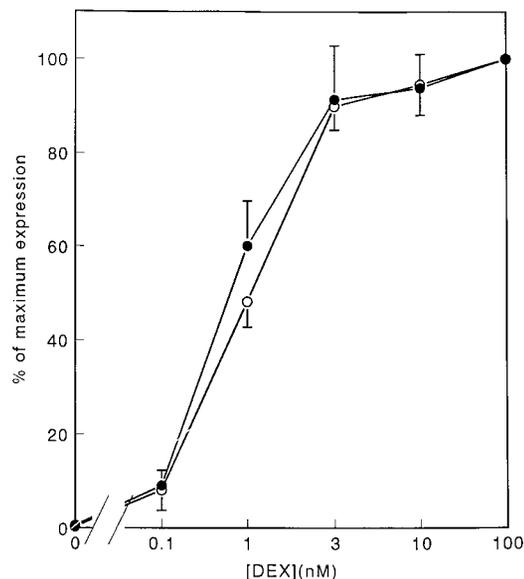


FIG. 4. Transcriptional activation of an MMTV-LUC reporter gene by the wild-type receptor (closed circles) and the codon 363 mutant (open circles). Data are the average of three separate transfection assays, each performed in duplicate. At concentrations of DEX between 0 and 100 nmol/L, the capacity of the N363S variant to activate the transcription from the MMTV promoter was unaltered, compared with the wild-type GR.

and after DEX administration. It has been recognized for a long time that GCs impair insulin-mediated glucose metabolism and induce insulin resistance (19). In this study, fasting insulin levels before DEX were similar in the two groups, but in response to the administration of 1 mg DEX, there was a significantly larger increase in the serum insulin levels in N363S carriers than in controls. After the administration of 0.25 mg DEX, there was a slight drop in insulin concentrations in the control group, whereas in the N363S carriers, mean insulin concentrations rose. It seems that in controls, 0.25 mg DEX is not sufficient to induce insulin resistance, whereas in the N363S carriers, this low dose of DEX forces the insulin concentrations to increase, to maintain a normal plasma glucose concentration. These data support the assumption that N363S carriers have an increased sensitivity to GCs.

With respect to the potential long-term effects of the codon 363 mutation, three parameters were investigated: BMI, blood pressure, and BMD. GCs are involved in the syndrome of abdominal obesity caused by stimulatory effects on lipid accumulation in adipose tissue (20). Patients with Cushing's syndrome have a centripetal fat distribution, resulting in a higher BMI. This seems to be caused by an increased lipoprotein lipase activity in combination with a lowered capacity for fat mobilization in the central regions (21). The net effect of hypercortisolemia is an increase at triglyceride storage in visceral adipocytes (22). At the second examination, the N363S carriers had a significantly higher BMI than controls, whereas at the first examination, there was a trend towards a higher BMI in the N363S carriers. The higher BMI observed in these persons supports the view that they are more sensitive to effects of GCs. There were, however, no

differences in blood pressure between the two groups, neither at the first nor at the second examination.

It is well known that patients under treatment with GCs show a reduced bone mass and an increased fracture risk. GCs have been found to influence bone and calcium metabolism at several levels (23, 24). The effects of GCs on BMD are more profound on trabecular bone than on cortical bone (24). The data from the present investigation indeed demonstrate a lower BMD in the lumbar spine of the N363S carriers, compared with control subjects. However, this difference in BMD did not reach statistical significance. In the femoral neck, there were no differences in BMD between the groups.

Other investigations previously have found the N363S polymorphism in a CR patient, who also showed a splice-site deletion (10), and in the small-cell long-cancer cell lines DMS-79 (25) and COR L24 (26). These authors found no significant difference in the capacity of this variant GR, relative to the wild-type, to activate transcription of the CAT gene in an MMTV-driven reporter construct after transfection into COS-1 cells. Our own results, using the more sensitive MMTV-LUC system, also do not indicate a difference between N363S and wild-type GR in this respect. Apart from transcriptional activation, GR also is involved in transcriptional repression of target genes. It does so either by binding directly to so-called negative glucocorticoid-responsive elements (27, 28) or via interactions with factors such as activating protein-1 (AP-1) (29, 30) or nuclear factor- $\kappa$ B (21–23), thereby preventing these factors from activating their target genes. Cotransfection experiments have shown that the codon 363 variant has an unaltered capacity to repress target genes via negative glucocorticoid-responsive elements or via the above mentioned transcription factors (31).

Our results of the whole-cell DEX-binding assays demonstrate no differences between the variant- and the wild-type GR in this respect, but because the mutation is located in the N-terminal part of the GR, no alterations in the number of receptors or the ligand binding capacity in peripheral mononuclear leucocytes from N363S carriers were expected. The mitogen-induced proliferation assay, on the other hand, reflects the cellular response to GCs (32). One would expect to find alterations in the suppressibility of mitogen-stimulated thymidine incorporation in cultured leucocytes from N363S carriers. Indeed, a trend towards lower IC<sub>50</sub> values in the N363S carriers, compared with controls, was observed; but these differences did not reach statistical significance, probably as a result of the wide range of values obtained in controls.

In summary, we have shown significant effects of the N363S mutation on changes in insulin and cortisol levels after the administration of DEX, indicating increased sensitivity to acutely increased GC levels. Long-term effects of increased sensitivity to endogenous GCs (which might be partly compensated by counterregulatory mechanisms) could be reflected in the trends towards increased BMI and decreased BMD in trabecular bone. In this connection, it is of interest that about 5% of the patients treated with GCs develop severe side effects soon after starting the therapy; N363S carriers might be included in this subpopulation. The apparent discrepancy between these *in vivo* effects and our inability to confirm this increased sensitivity to GCs *in vitro*

is likely to be caused by the earlier-mentioned broad spectrum of regulatory mechanisms in which GCs and their receptors are involved. We showed earlier that GR mutations can effect transcriptional activation and repression differently (31). It could well be that COS-1 cells lack the transcription factors determining the effect of the N363S mutation *in vivo* and that this is an impediment for the study of subtle effects of this mutation *in vitro*.

Finally, our results may indicate that this polymorphism is linked to an additional genetic alteration in a gene of which the product is involved in the observed phenotypic differences. This yet-unknown factor might interact with the GR protein to exert its effect. However, for diagnostic purposes, the recognition of a point mutation in the GR gene linked to a phenotypic difference may prove very useful, whether this may solely, in part, or not at all be a consequence of altered GR function. A next step will be to investigate whether the codon 363 polymorphism might predict an increased sensitivity to the development of early and/or serious side effects during therapy with GCs.

In conclusion, our findings show that *in vivo*, the codon 363 variant of the GR is associated with an increased sensitivity to GCs in the direct response to exogenously administered DEX. BMI and BMD might also be effected by this variant GR. The molecular basis for this increased sensitivity remains to be elucidated.

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