CLINICAL STUDY

Glucocorticoid receptor gene haplotypes are not associated with birth anthropometry, blood pressure, glucose and insulin concentrations, and body composition in subjects born small for gestational age

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Abstract

Objective: Smaller size at birth has been associated with an increased risk of metabolic and cardiovascular disorders in adult life. Fetal programming of the hypothalamic–pituitary–adrenal axis has been suggested as a possible explanation. Fetal glucocorticoid (GC) overexposure has effects that suggest a role of GCs in this programming. The effects of GCs are mediated through the GC receptor (GR or NR3C1). Several functional polymorphisms have been described, which are associated with relative GC resistance or hypersensitivity. Our aim is to compare frequencies of GR haplotypes, characterized by the R23K, N363S, Bcl1, or 9β polymorphisms, in subjects born small for gestational age (SGA) and associate birth anthropometry data, response to GH treatment, blood pressure, glucose and insulin concentrations, and body composition with these haplotypes.

Design: In total, 418 SGA subjects and 697 healthy controls were enrolled in this study.

Methods: Anthropometry data were obtained, as well as blood samples to determine fasting glucose and insulin concentrations. Dual energy X-ray absorptiometry scans were used to measure the amount of fat and lean mass.

Results: No differences were found between GR haplotype frequencies in SGA children compared with healthy controls. No associations were found between GR haplotypes and birth length and birth weight, growth response during GH treatment, blood pressure, glucose and insulin concentrations, and body composition.

Conclusion: GR haplotypes and their effect on GC sensitivity do not seem to play a significant role in GH-induced catch-up growth and the risk factors of developing metabolic and cardiovascular disorders in adult life of SGA children.

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Introduction

Small size at birth has been associated with an increased risk of metabolic and cardiovascular disorders in adult life, such as hypertension, hyperlipidemia, insulin resistance, type 2 diabetes mellitus, and osteoporosis (1, 2). Therefore, children born small for gestational age (SGA), defined as birth length or birth weight < -2 s.d. for gestational age, might have an increased risk of metabolic and cardiovascular disorders in adult life. Only 2.3% of all children born live are SGA (3). Postnatal catch-up growth in weight or body mass index (BMI) is also a risk factor for hypertension, ischemic heart disease, insulin resistance, and obesity in later life (4–6). Altered fetal programming of the

hypothalamic–pituitary–adrenal (HPA) axis has been suggested as an explanation for this association (7). There are several effects of fetal glucocorticoid (GC) overexposure that suggest a role in early life programming of adult cardiovascular and metabolic disorders. Exogenous GCs lead to fetal growth retardation and a lower birth weight in humans. Human fetal blood cortisol concentrations are increased in infants after intrauterine growth retardation and also in preeclampsia, implicating higher endogenous cortisol concentrations in retarded fetal growth (1, 7). Moreover, the major systems affected by early life programming are GC sensitive, namely blood pressure and blood glucose/insulin resistance (1). Disorders such as hypertension and insulin resistance are also present in Cushing's

syndrome, which is caused by GC excess (8). All these findings together suggest a role of GCs in the early life programming of adult disorders.

The effects of GCs are mediated through the GC receptor (GR or NR3C1). The GR is a member of the nuclear receptor family and is expressed in most fetal tissues from the early embryonic stages (1, 7). The individual GC sensitivity differs between subjects and might be partly explained by polymorphisms of the GR gene. Four GR polymorphisms are thought to be physiologically functional: R23K (rs6189 and rs6190), N363S (rs6195), Bcl1 (rs41423247), and the 9 β (rs6198) polymorphism. They are associated with either GC hypersensitivity or resistance. GC hypersensitivity is associated with clinical signs such as visceral obesity, type 2 diabetes mellitus, higher BMI, and hypertension and GC resistance is associated with increased length, increased muscle mass, and a healthier metabolic profile.

The R23K polymorphism consists of two linked single nucleotide polymorphisms in codons 22 and 23 in exon 2. The alteration at the DNA level is GAG AGG to GAA AAG change, which leads to a glutamic acid–arginine (E–R) to glutamic acid–lysine (E–K) change (9). This polymorphism is associated with a relative GC resistance, a healthier metabolic profile, and increased insulin sensitivity (10, 11).

The N363S and the Bcl1 polymorphisms are associated with increased sensitivity to GCs, visceral obesity, and type 2 diabetes. The N363S polymorphism is located in codon 363 and causes an AAT $\rightarrow$ AGT nucleotide change. This change results in an amino acid change from asparagine to serine. The Bcl1 polymorphism is a C $\rightarrow$ G substitution located in intron 2 (12). The N363S and Bcl1 polymorphism are both associated with higher BMI, higher waist-to-hip ratio, visceral adiposity, hypertension, and higher cholesterol and triglyceride concentrations.

The GR-9 β polymorphism is an ATTTA to GTTTA change in exon 9 β , resulting in an increased expression and stability of GR- β *in vivo*, consequently leading to GC resistance (13–15). GR-9 β has been associated with decreased GC transrepressive activity (16), decreased microbial colonization (17), and with increased inflammatory mediators leading to an increased risk to cardiovascular disease (18).

We hypothesized that there could be an association between the GR polymorphisms, small size at birth, and risk factors for metabolic and cardiovascular disorders in later life. We set out to study this in a group of subjects born SGA compared with controls.

Subjects and methods

Subjects

This study included 418 subjects (196 males and 222 females) born SGA. All subjects fulfilled the following inclusion criteria: i) birth length and/or birth weight

SDS below -2.0 s.d. for gestational age (19); ii) an uncomplicated neonatal period, without signs of severe asphyxia, sepsis, or long-term complications of respiratory ventilation. Exclusion criteria were twins, non-Caucasian ethnicity, endocrine or metabolic disorders, and chromosomal defects or syndromes. The cause of SGA in this study population is unknown. Catch-up growth was defined as an achieved height SDS for age above -2.0 s.d. according to Dutch standards at last visit (20). SGA children who did not show catch-up growth (SGA short, $n=191$), started with GH treatment. The mean age at start of treatment was 7.51 (2.9) years. Children were treated with biosynthetic GH at a dose of 1 mg/m² body surface area/day. The GH dosage was adjusted to the calculated body surface every 3 months. For the present study, the growth during 1 year of GH treatment was analyzed. Written informed consent was obtained from the parents/guardians of each child. This study was approved by the Medical Ethics Committee of the Erasmus Medical Center.

Control group

The control group consisted of 165 healthy subjects who were randomly collected from hospitals in The Netherlands, where they had gone because of a minor accidental health problem, but otherwise they were normal. All participants were born appropriate for gestational age and had normal adult height. To compare polymorphism frequencies, we also used blood samples from 532 healthy subjects who were randomly collected from the Rotterdam blood donation bank. Written informed consent was obtained from all the participants.

Anthropometric analysis

Data on gestational age, birth length, and birth weight were obtained using primary health care records and hospital records. In all subjects, standing height was measured to the nearest 0.1 cm by a Harpenden stadiometer and weight was measured to the nearest 0.1 kg by scale (Servo-Balance KA-20-150S) wearing underwear only. Standard height was expressed as SDS adjusting for sex and age using Dutch standards (20). Body weight was expressed as SDS adjusting for sex and height using Dutch standards (20).

Blood pressure, insulin, and glucose analysis

In 317 SGA subjects, systolic and diastolic blood pressure was measured every 5 min during a period of 1 h in the morning. Mean systolic and diastolic blood pressure was calculated from these measurements. In 255 SGA subjects, fasting glucose concentrations were measured and in 155 SGA subjects, fasting insulin concentrations were also determined.

Body composition analysis

Fat and lean body mass distributions were measured with dual energy X-ray absorptiometry scans (DEXA; type Lunar DPX-L; GE Healthcare, Madison, WI, USA) in 243 SGA subjects. Total lean body mass, total fat body mass, and fat percentages were determined.

Genetic analysis

DNA was isolated from the peripheral blood leukocytes using standard techniques and dissolved in double-distilled water and stored at -20°C . Allelic discrimination was performed to genotype the subjects, using TaqMan Universal PCR master mix, primers and probes (Applied Biosystems), and a Taqman ABI Prism 7900HT Sequence Detection System as previously described (17). 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 for each polymorphism. The polymorphisms used in this study were chosen on the basis of their reported functionality, and not as tagging SNPs. In addition, we constructed haplotypes as previously described (17). Haploview showed that four of the five haplotypes that were constructed cover around one-third of the GR gene variability (19 out of 57 haplotypes). The Bcl1 polymorphism is not available in the HapMap database, therefore the haplotype characterized by this polymorphism could not be taken into account. However, the minor allele of this polymorphism has the highest minor allele frequency. This suggests that a considerable part of the variation in

Table 1 Baseline characteristics. Data are expressed as median (25th to 75th percentiles).

	SGA
Number	418
Male (%)	46.9
Gestational age (weeks)	38.00 (36–40)
Birth length SDS	−2.74 (−3.79 to −2.24)
Birth weight SDS	−2.35 (−3.06 to −1.91)
Age (years)	10.53 (6.74–19.96)
Height SDS	−2.65 (−3.33 to −1.78)
Weight SDS	−2.76 (−3.57 to −1.74)
Body mass index SDS ^a	−1.11 (−1.90 to −0.30)
Diastolic blood pressure (mmHg)	62.54 (56.00–67.46)
Systolic blood pressure (mmHg)	107.92 (99.00–115.00)
Insulin (mmol/l)	7.33 (5.00–11.00)
Glucose (mmol/l)	4.80 (4.50–5.13)
Fat percentage	15.03 (8.17–24.50)
Fat mass (g)	6221 (2042–14 898)
Lean mass (g)	36 119 (18 182–46 257)

^aBody mass index SDS adjusted for age.

the GR gene is covered by the haplotypes that we have constructed (21). The GR gene and haplotypes are shown in Fig. 1.

Statistical analysis

All statistical analyses were performed with SPSS (version 16.0.1 for Windows, SPSS Inc., Chicago, IL, USA). Comparison of GR haplotypes frequencies between the SGA group and the control group was performed with χ^2 test. Comparison of the means of anthropometric measures, blood pressures, fasting glucose and insulin concentrations, total fat mass, fat percentage, and total lean mass in the SGA group with the genotypes was performed using the ANCOVA test. All analyses were corrected for age and sex. A P value <0.05 was considered to indicate a significant difference.

Figure 1 Schematic overview of the glucocorticoid receptor (GR) gene polymorphisms and genotypes. The alleles were defined as haplotypes such as G-A-C-A (wild-type) representing a guanine (G) nucleotide at the 200 G>A polymorphic site, an adenosine (A) nucleotide at the 1220 A>G polymorphic site, a cytidine (C) nucleotide at the C>G Bcl1 polymorphic site, and an adenosine (A) at the A>G 9β polymorphic site. TAD, transactivating domain; DBD, DNA-binding domain; LBD, ligand-binding domain.

Results

Baseline characteristics of 418 SGA subjects are shown in Table 1. In SGA subjects and controls, the distribution of genotypes for all individual GR gene polymorphisms was in Hardy–Weinberg equilibrium ($P>0.05$). No significant difference was found in allele frequencies (Table 2). No significant differences in birth length and birth weight in the total SGA group were found between the four GR haplotypes, except for haplotype 4. Carriers of this haplotype had lower birth weight SDS ($P=0.044$; Table 3). Analysis of the effect of GH treatment in SGA subjects who did not show catch-up growth (SGA short), measured as gain in height SDS after 1 year of GH treatment, did not show differences in height gain between GR haplotypes (Table 3).

Mean systolic and diastolic blood pressure and fasting insulin and glucose concentrations did not show any significant difference between the GR haplotypes

Table 2 Glucocorticoid receptor gene haplotype frequencies in small for gestational age (SGA) subjects and healthy controls.

Genotype	SGA		Controls ^a		P value
	n	%	n	%	
Haplotype 0					0.098
0	154	38.4	245	35.8	
1	160	39.9	317	46.3	
2	87	21.7	123	18.0	0.648
Haplotype 1					
0	385	93.2	642	92.2	
1	28	6.8	53	7.6	0.330
2	0	0.0	1	0.1	
Haplotype 2					
0	380	91.3	646	92.9	0.251
1	36	8.7	49	7.1	
Haplotype 3					
0	175	42.2	261	37.4	0.265
1	188	45.3	333	47.8	
2	52	12.2	103	14.8	
Haplotype 4					
0	286	71.3	519	75.8	
1	103	25.7	150	21.9	
2	12	3.0	16	2.3	

P values are calculated with χ^2 test; 0, noncarriers; 1, heterozygous carriers; 2, homozygous carriers; n, number of SGA subjects.

^aControls include samples from the Rotterdam blood bank.

(Table 4). In 243 SGA subjects, fat percentage, total fat mass, and total lean mass were measured using DEXA scans. No differences were found in mean fat percentage, mean total fat mass, and mean total lean mass between the five GR haplotypes (Table 5). These results were adjusted for age, sex, and height.

When divided between SGA subjects who did not reach normal height (SGA short) and subjects who showed catch-up growth (SGA-CU), also no differences

in allele frequencies were found: haplotype 0 showed a heterozygous frequency of 37.6% in SGA short versus 41.9% in SGA-CU and a homozygous frequency of 22.4 vs 16.2% ($P=0.230$). Haplotype 1 showed a heterozygous frequency of 6.4% in SGA short versus 8.3% in SGA-CU ($P=0.518$). Haplotype 2 showed a heterozygous frequency of 7.8 vs 10.1% ($P=0.470$) and haplotype 3 showed a heterozygous frequency of 43.7 vs 52.3% and a homozygous frequency of 10.4 vs 13.8% ($P=0.100$). Haplotype 4 showed a heterozygous frequency of 27.5 vs 22.9% and a homozygous frequency of 3.5 vs 2.9% ($P=0.668$).

Discussion

In this study, we found no differences in five GR haplotype frequencies in the SGA subjects compared with healthy controls. In addition, between GR haplotypes, no difference was found in mean birth weight and birth length, except for the carriers of haplotype 4, characterized by 9 β , who showed lower birth weight SDS than the noncarriers. We found no differences in growth response during 1 year of GH treatment among the GR haplotypes in SGA short subjects. In addition, no significant differences were found in blood pressure, fasting insulin and glucose concentrations, percentage fat and total fat, and lean mass between the GR haplotypes.

GR gene polymorphisms are known to play a role in the sensitivity to GCs (12). Several studies suggest that fetal programming of the HPA axis might result in pathology in later life in SGA children. Subjects born with low birth weight have higher plasma cortisol

Table 3 Differences in mean birth length SDS, birth weight SDS in the total group of small for gestational age (SGA) subjects, and height gain SDS after 1 year of GH treatment in SGA short subjects between the glucocorticoid receptor gene haplotypes.

Genotype	n	Birth length	P value	n	Birth weight	P value	n	Height gain after 1 year GH treatment	P value
Haplotype 0			0.468			0.337			0.162
0	153	-3.20		158	-2.49		71	0.98	
1	148	-3.04		156	-2.43		67	1.17	
2	84	-3.21	0.070	87	-2.30	0.178	46	1.27	0.684
Haplotype 1									
0	371	-3.10		385	-2.40		176	1.14	
1	26	-3.57	0.695	28	-2.65	0.678	13	1.04	0.291
Haplotype 2									
0	365	-3.12		380	-2.42		174	1.10	
1	35	-3.21	0.362	36	-2.35	0.563	17	1.32	0.168
Haplotype 3									
0	168	-3.13		175	-2.38		92	1.23	
1	181	-3.19	0.545	188	-2.46	0.044	77	1.05	0.376
2	50	-2.90		52	-2.32		21	0.90	
Haplotype 4									
0	273	-3.12		286	-2.36		126	1.15	
1	101	-3.22		103	-2.62		54	1.02	
2	11	-2.81		12	-2.29		4	1.54	

Data are expressed as means, P value was calculated using ANCOVA; 0, noncarriers; 1, heterozygous carriers; 2, homozygous carriers; n, number of SGA subjects.

Table 4 Differences in systolic and diastolic blood pressure (mmHg), fasting glucose (mmol/l), and fasting insulin (mmol/l) concentrations between glucocorticoid receptor gene haplotypes in small for gestational age (SGA) subjects.

Genotype	n	Systolic blood pressure	P value	Diastolic blood pressure	P value	n	Fasting glucose	P value	n	Fasting insulin	P value
Haplotype 0			0.734		0.434			0.377			0.134
0	119	107.92		61.94		98	4.94		60	9.20	
1	186	107.69		62.27		149	4.83		91	8.08	
Haplotype 1			0.313		0.647			0.603			0.918
0	294	107.53		62.13		238	4.88		145	8.43	
1	20	110.07		62.81		16	4.98		10	8.90	
Haplotype 2			0.142		0.131			0.758			0.743
0	288	108.04		62.36		234	4.89		140	8.43	
1	29	104.53		59.76		21	4.82		15	8.74	
Haplotype 3			0.858		0.672			0.990			0.570
0	134	107.63		62.08		100	4.87		64	8.16	
1	182	107.87		62.14		155	4.89		91	8.68	
Haplotype 4			0.668		0.470			0.379			0.331
0	217	107.64		61.97		185	4.90		112	8.31	
1	88	108.11		62.57		62	4.80		39	9.17	

Data are expressed as means, *P* value was calculated using ANCOVA; data are adjusted for age and gender; 0, noncarriers; 1, heterozygous and homozygous carriers; *n*, number of SGA subjects.

concentrations in adult life, suggesting altered HPA axis programming (7). Individual differences in GC sensitivity might play a role in fetal programming of the HPA axis. Based on the reported higher cortisol concentrations, one could expect that SGA subjects have a higher frequency of GR haplotypes associated with relative GC resistance (haplotype 1, characterized by the R23K and the 9 β polymorphism and haplotype 4, characterized by the 9 β polymorphism), since this relative GC resistance would be compensated by increased cortisol concentrations. We did not find this association. Our pilot study in a group of 119 SGA children showed a lower frequency of the Bcl1 polymorphism than the controls (22). A previous cohort study in the general population

showed that carrying a GR haplotype including the Bcl1 polymorphism was associated with a smaller size at birth corrected for gestational age. Carriers of this haplotype had both a lower birth weight and birth length than noncarriers (2). In contrast, Bertalan *et al.* (23) found that the Bcl1 polymorphism was associated with higher gestational-age-adjusted birth weight in preterm neonates. We studied a large group of SGA subjects and did not find an association between the haplotype 3, which includes the Bcl1 polymorphism, and size at birth corrected for gestational age. This is in line with the results published by Geelhoed *et al.* (21). They showed that in a large population-based cohort, GR polymorphisms were not associated with birth

Table 5 Differences in fat percentage, total fat mass (g), and total lean mass (g) between glucocorticoid receptor gene haplotypes in small for gestational age (SGA) subjects.

Genotype	n	Fat percentage	P value	Total fat mass	P value	Total lean mass	P value
Haplotype 0			0.798		0.544		0.716
0	90	19.13		4808		30 761	
1	141	17.27		5082		30 620	
Haplotype 1			0.446		0.716		0.468
0	225	17.81		4932		30 479	
1	17	16.75		4645		29 854	
Haplotype 2			0.995		0.816		0.353
0	223	17.68		4932		30 339	
1	20	18.79		5117		31 046	
Haplotype 3			0.369		0.494		0.844
0	93	16.54		5164		30 549	
1	149	18.58		4864		30 479	
Haplotype 4			0.647		0.971		0.615
0	174	17.99		4977		30 620	
1	57	18.00		4966		31 477	

Data are expressed as geometric means; *P* value was calculated using ANCOVA; data are adjusted for age, gender, and height; 0, noncarriers; 1, heterozygous and homozygous carriers; *n*, number of SGA subjects.

weight, birth length, and postnatal growth (21). Interestingly, we found an association between heterozygous carriers of haplotype 4, characterized by the 9 β polymorphism and lower birth weight corrected for gestational age. The 9 β polymorphism is located at the 3'-UTR end of the *GR* gene, leading to increased stability of the GR- β splice variant at the mRNA level. This variant functions as a dominant negative inhibitor of the active GR- α isoform, resulting in a relative GC resistance. Carriers of the 9 β polymorphism seem to have a decreased GC transrepression with a normal transactivation (16). For haplotype 4, characterized by the 9 β polymorphism, no associations were found with cortisol response in dexamethasone-suppression test, BMI, waist-to-hip ratio, insulin sensitivity, and lipid profile (16). Haplotype 4 was associated with decreased transrepressive effects on the immune system and inflammation. Carriers of haplotype 4 had increased cardiovascular risk factors, namely elevated sensitive C-reactive protein and interleukin 6 concentrations and increased intima media thickness, resulting in increased risk of myocardial infarction and coronary heart disease (18). Our finding of lower birth weight in heterozygous carriers of haplotype 4 than noncarriers is in line with the *a priori* hypothesis based on mechanistic background and phenotype of haplotype 4. However, the difference in birth weight between carriers and noncarriers of haplotype 4 is not highly significant and shows no allele-dosage effect. Therefore, we do not interpret this finding as a realistic association until future studies can reproduce this.

SGA children showing postnatal catch-up in BMI have a higher risk of developing insulin resistance, central obesity, and cardiovascular diseases (24–26). Those with catch-up in BMI had lower insulin sensitivity than SGA children without catch-up (26–28). Therefore, we expected a higher frequency of GR haplotypes leading to GC hypersensitivity (haplotype 2, characterized by the N363S polymorphism and haplotype 3, characterized by the Bcl1 polymorphism) in SGA children with catch-up growth. Our subgroup analysis of SGA children with short stature versus those with catch-up growth showed no differences between the GR haplotypes. Surprisingly, we did not find an association between the GR haplotypes and the catch-up growth or the insulin resistance. This might be the result of the relatively small study groups. However, other studies did find associations in smaller subgroups than ours (11, 29–31).

The growth response during 1 year of GH treatment was not different between carriers of the various GR polymorphisms. In a previous study, preterm born carriers of the 23K variant of the R23K polymorphism showed complete catch-up growth to an adult height similar to the reference mean (32), indicating that the R23K polymorphism might be beneficial for growth. As a result, we expected more gain in height SDS during GH treatment in carriers of haplotype 1, including the R23K and the 9 β polymorphism, but we could not

confirm this expectation. This suggests that GR gene polymorphisms do not have a substantial effect on the sensitivity to GH in this group. The growth retarding effect of GCs, known from corticosteroid treatment, is caused by the inhibition of endogenous GH secretion, collagen synthesis, cartilage sulfation, chondrocyte mitosis, GH receptor binding, and insulin-like growth factor 1 activity (33). It should, however, be noted that growth retardation is only seen when supraphysiological doses of corticosteroids are used.

We also found no differences in systolic and diastolic blood pressure, fasting glucose and insulin concentrations, and body composition between the GR haplotypes in SGA subjects. In the study published by Finken *et al.* (32), preterm born carriers of the R23K polymorphism had lower fasting insulin concentrations than noncarriers, but systolic and diastolic blood pressure and body composition measured by waist-to-hip ratio, absolute fat mass, and fat percentage also showed no differences between carriers and noncarriers of the R23K or N363S polymorphism in preterm born children (32).

Recently, subgroups within the SGA group have been described in a study by Ester *et al.* (34). Differences in catch-up growth were found between SGA children with only small birth length, SGA children with a small birth length and a low birth weight, and SGA children with a small birth length, a low birth weight, and a small head circumference (34). In the future it might be interesting to investigate whether GR gene polymorphisms have different effects in these subgroups.

In conclusion, we found no differences in allele frequencies of the GR gene haplotypes between SGA subjects and healthy controls and no associations were found with birth anthropometry, response to GH treatment, blood pressure, fasting glucose and insulin concentrations, and body composition. Therefore, GR haplotypes do not seem to play a major role in body composition, GH-induced growth, glucose and insulin concentrations, and blood pressure in SGA subjects.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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