CLINICAL STUDY

Investigation of glucocorticoid receptor polymorphisms in relation to metabolic parameters in Addison's disease

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Abstract

Background: Uncertainty exists whether glucocorticoid receptor (GCR) polymorphisms play a role in steroid-related side effects in Addison's disease (AD) patients on hydrocortisone. The polymorphisms *Bcll* and N363S appear to increase sensitivity to cortisol, while the ER22/23EK polymorphism has been associated with resistance to cortisol.

Method: One hundred and forty seven AD patients, and gender, and ethnicity-matched controls were recruited in South Africa. Three polymorphisms in the GCR were studied, using PCR followed by restriction fragment length analysis. Associations with BMI, lipids, glucose and inflammatory markers were investigated.

Results: In both patients and controls, the *Bcll* polymorphism occurred more frequently in whites than in other ethnic groups studied but was not associated with any of the metabolic parameters tested. The ER22/23EK polymorphism was associated with an increased BMI in both patients (29.4 vs 24.7 kg/m²) and control subjects (26.3 vs 24.2 kg/m²). The ER22/23EK polymorphism was also associated with lower LDL cholesterol in control subjects (3.46 vs 3.93 mmol/l) and in patients (3.52 vs 4.10 mmol/l). N363S was associated with increased BMI in controls 29.9 kg/m² vs wild type 24.8 kg/m². Median hydrocortisone doses were greater in patients heterozygous for either ER22/23EK 30.0 mg or N363S 25.0 mg polymorphisms than in wild type patients 20.0 mg (both comparisons).

Conclusion: Alterations in lipids, BMI and hydrocortisone dose were associated with two polymorphisms. Further larger studies are warranted to corroborate these findings.

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Introduction

Addison's disease (AD) patients require lifelong hydrocortisone replacement therapy, but uncertainty exists concerning ideal hydrocortisone doses. Despite physicians' assessments that hydrocortisone supplementation is adequate, many AD patients report subjective impaired health quality and increased doses are often prescribed, leading to potentially deleterious effects (1, 2, 3, 4, 5, 6). Elevated total cholesterol (TC) (7, 8, 9, 10), raised triglycerides (TGs) (11, 12), increased LDL cholesterol (LDLC) (8, 9) and reduced HDL cholesterol (HDL) (9) were found in association with glucocorticoids, potentially explaining at least in part why the mortality rate from cardiovascular disease (CVD) is twofold increased in some studies of AD (2).

Cortisol exerts multiple pleiotropic actions critical for metabolic, physiological and stress-related conditions (13). The human glucocorticoid receptor (GCR) gene has nine exons, located on chromosome 5 (5q31) (14).

GCR polymorphisms are found throughout the GCR gene and their impact on function is variable, ranging from no impact to either increased or decreased cortisol sensitivity. The *Bcll* and N363S polymorphisms are reported to increase cortisol sensitivity. Clinical outcomes reported with increased cortisol sensitivity include an increase in BMI of 1.4 kg/m² in heterozygotes and 2.0 kg/m² in homozygotes in young people (15) for the *Bcll* polymorphism and an increase in LDLC (16), TG (16, 17), TC (17, 18), as well as a higher BMI (19, 20) with the N363S polymorphism. The ER22/23EK polymorphism decreases cortisol sensitivity, correlating with a reduction in TC (21), LDL (21) and a lower BMI during pregnancy (22). Altered glucocorticoid sensitivity associated with GCR polymorphisms is a consequence of a modified transcript (23).

Little information exists regarding the role that the GCR polymorphisms may play in the development of glucocorticoid-related side effects in patients receiving hydrocortisone replacement and whether empiric doses

of hydrocortisone need to be altered in the presence of certain polymorphisms. For example, the ER22/23EK polymorphism is associated with a degree of glucocorticoid resistance (21), yet it is unknown whether increased doses of hydrocortisone are required. If deleterious metabolic consequences are identified in association with the sensitising GCR polymorphisms *Bcll* (24) and N363S (25, 26), genotyping followed by adjustment of hydrocortisone dose according to the genotype may aid management of patients with AD. The aim of this study was to investigate the associations between certain GCR polymorphisms and metabolic and inflammatory markers in groups of AD patients and healthy controls.

Materials and methods

Subjects and methods

This study was approved by the University of Cape Town Human Research and Ethics Committee and all participants signed informed consent. We included 147 AD patients and 147 age-, gender- and ethnicity-matched healthy controls who attended a volunteer blood donor clinic. AD patients were recruited from a South African national database, in which all medical practitioners were asked to register patients with AD under their care. Clinical data were extracted from patients' notes and patient interviews. Blood was taken in a non-fasting state for DNA extraction, plasma lipid determination and measurement of CVD risk markers.

Hydrocortisone dose

Hydrocortisone doses were modified by the patients' treating physicians based on symptoms suggestive of either hypoadrenalism or cortisol excess. There were no standard protocols to modify replacement doses. The majority of patients received a dose of ~20 mg hydrocortisone per day, irrespective of body weight. As this was an observational study, the authors had no influence on hydrocortisone dosing. Only ambulatory, well patients with AD on stable doses of hydrocortisone replacement for a minimum of 3 months were included in this study. Careful review of concomitant medication was undertaken to ensure that the hydrocortisone dose was not influenced by drugs known to induce or inhibit hydrocortisone metabolism.

Assays for lipids, lipoproteins and markers of cardiovascular risk

Assays for TG, TC, non-esterified fatty acids (NEFA) and random blood glucose (RBG) were carried out with commercially available enzymatic kits, using standard curves and calibrators. The respective kits for TG, TC, NEFA and RBG were KAT TGs (category number T801-

glycerol-3-phosphate-oxidase method), KAT cholesterol (category number CH704-cholesterol-oxidase method), Roche free fatty acids (category number 11383175001-acyl-CoA-synthetase) and KAT glucose (category number GP747-glucose-oxidase method). HDLC was measured following the first step in the Gidez assay (27), which yields HDLC in the supernatant of a heparin-Mn precipitation of apoB-containing lipoproteins. LDL particle size was measured by non-denaturing gradient gel electrophoresis. LDLC was calculated by the Friedewald equation (28).

Highly sensitive C-reactive protein (hs-CRP) was measured using an immunoturbidimetric assay (Roche Diagnostics GmbH), demonstrating a coefficient of variation of 4 and 3% at serum concentrations of 1 and 15 mg/l respectively. Framingham risk was calculated using an algorithm that includes diabetes as a risk factor so that comparisons could be made across the entire cohort (29). Currently, diabetes is regarded as a secondary prevention equivalent for CVD and is not included as a risk factor in the Framingham algorithm any longer (30).

DNA extraction

Genomic DNA purification was performed using a Promega wizard kit, as published previously (31).

Detection of the GCR single nucleotide polymorphisms: *Bcll*, ER22/23EK and N363S

Genotyping of the GCR gene for detecting the single nucleotide polymorphism (SNP) changes *Bcll* G/C (RS41423247), ER22/23EK AA/GG (rs6189) and N363S G/A (rs6195) was performed by PCR amplification, followed by restriction fragment length analysis. Primers were designed using Oligo.exe (National Biosciences, Plymouth, MN, USA). Primer pairs used in the PCR amplification are shown in Table 1. Each PCR contained ~100–500 ng/μl genomic DNA, 1×PCR buffer, 1.5 mM MgCl₂, 200 μM dNTP and 15 pM of each primer 0.75 U Taq DNA polymerase (Promega-Go Taq Flexi DNA polymerase) in a final volume of 30 μl. PCR conditions for all the reactions were the same. The denaturation step was performed at 98 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s and annealing was carried out at 52 °C for 30 s. Elongation occurred at 72 °C for 30 s and a final extension occurred at 72 °C for 8 min. rs41423247 PCR products were digested using *Bcll* restriction enzyme (C allele = 146 + 86 bp, G allele = 232 bp), rs6189 PCR products were digested using *MnI* restriction enzyme (GAG AGG allele = 201 + 143 + 50 + 35 + 15 + 11 + 3 bp, GAA AAG allele = 201 + 178 + 50 + 11 + 3 bp) and rs6195 PCR products were digested using *Tsp* 5091 (A allele = 91 + 36 bp, G allele = 127 bp). Products were separated on an agarose gel, visualised with ethidium bromide and fragment sizes were compared with standard markers (1 kb plus (Promega)).

Table 1 Primer pairs used for the detection of SNPs of the GCR.

Polymorphism	SNP	Primer sequences
<i>Bcl</i> G/C	rs41423247	F: 5'-GCA GTG AAC AGT GGT ACC AGA CC-3' R: 5'-AAA GAG AAA AAT CAA ACG AA GC-3'
ER22/23EK AA/GG	rs6189	F: 5'-GCT GCC TCT TAC TAA TCG GAT CA-3' R: 5'-TTT AAG TCT GTT TCC CCC GAG-3'
N363S G/A	rs6195	F: 5'-TTT AAT GTC ATT CCA CCT ATT CCC-3' R: 5'-AGC CAT TAG AAA AAA CTG TTC GAC-3'

GCR, glucocorticoid receptor; SNP, single nucleotide polymorphism; rs, reference SNP.

Genotyping quality was verified using the Hardy–Weinberg equilibrium (HWE) calculations on the controls, with none of the genotypes deviating from HWE, as shown by their respective *P* values in parentheses: *Bcl* (0.11), N363S (1.00) and ER22/23EK (1.00). Samples were not sequenced for other GCR polymorphisms.

Statistical analysis

Logistic regression was used for all comparisons between patients and controls, enabling us to control for possible confounders such as age and gender. Characteristics and polymorphism prevalence were compared between patients and controls, adjusting for age, gender and ethnicity (unadjusted comparisons were made for adjustment variables). We used general linear models to investigate whether the polymorphism had an effect on numerical characteristics or phenotypes (like TG or TC, transformed to symmetry when required) in healthy controls and in patients. Logistic regression models were used to assess the effect of the polymorphisms on dichotomous characteristics such as the presence or absence of small dense LDL particles. In healthy controls, these analyses were adjusted for BMI, age, gender and ethnicity, and for patients, the analyses

were adjusted for BMI, age, gender, ethnicity, foreign ancestry and hydrocortisone dose, where possible. Further analyses assessed whether the effect of the polymorphisms differed between patients and controls (interaction). Interaction analyses were adjusted for BMI, gender, age and ethnicity. We repeated the analyses of all lipid parameters after excluding all patients on lipid-lowering therapy and also noted an extreme outlier amongst the TSH results (31.2 mIU/l) in a patient who was non-compliant with thyroxine replacement and excluded this value from analyses of the TSH data set. 'R: A language and environment for statistical computing', freely available from <http://www.R-project.org/> and R package genetics, was used for all statistical analyses. We regarded *P* values below 0.05 as statistically significant. For the polymorphisms with low frequency, only descriptive data are shown.

Results

Patients and controls were well matched for gender and ethnicity but patients had lower BMI. Patients had more atherogenic lipid profiles, as evidenced by a preponderance of small dense LDL, higher TG, lower HDLC and higher hs-CRP levels (Table 2). None of the

Table 2 Clinical characteristics in Addison's disease patients and controls.

Clinical characteristics	Patients (<i>n</i> =147)	Controls (<i>n</i> =147)	<i>P</i> value
Age (years), median (IQR)	46.0 (33.5–61.0)	41.5 (33.0–53.0)	0.108
Gender (<i>n</i> (%))			
Female	90 (61)	90 (61)	1.000
Male	57 (39)	57 (39)	
Ethnicity (<i>n</i> (%))			
White	97 (66)	97 (66)	1.000
Mixed ancestry	34 (23)	34 (23)	
Asian	5 (3)	5 (3)	
Black	11 (7)	11 (7)	
BMI (kg/m ²), median (IQR)	24.7 (22.1–30.3)	26.4 (24.1–31.2)	0.001
TG (mmol/l), median (IQR)	1.67 (1.1–2.61)	1.39 (0.97–2.13)	0.011
TC (mmol/l), mean (s.d.)	5.70 (1.55)	5.77 (1.26)	0.226
HDLC (mmol/l), median (IQR)	0.78 (0.53–1.07)	1.08 (0.93–1.27)	<0.001
LDLC (mmol/l), mean (s.d.)	4.07 (1.37)	3.89 (1.17)	0.599
Small dense LDL (<i>n</i> (%))	17/147 (12)	5/147 (3)	0.001
NEFA (μmol/l), median (IQR)	341.0 (142.5–654.0)	467.0 (325.8–644)	<0.001
Lipid-lowering therapy (<i>n</i> (%))	19/147 (13)	0 (0)	
hs-CRP (mg/l), median (IQR)	2.2 (0.97–6.38)	1.5 (0.64–3.25)	0.024

TG, triglyceride; HDLC, HDL cholesterol; LDLC, LDL cholesterol; NEFA, non-esterified fatty acid; IQR, interquartile range. *P* value: comparison between patients vs controls, adjusted for age, gender, race and BMI. Statistically significant differences are in boldface.

controls were on lipid-lowering therapy, in contrast to 19 (13%) of the patients.

Hydrocortisone dose

The median (interquartile range) dose of hydrocortisone adjusted for body weight was 0.33 (0.25–0.45) mg/kg. Most patients were taking doses between 0.2 and 0.4 mg/kg, but a few were taking substantially higher doses. We detected no significant correlation between total daily hydrocortisone dose and duration of AD ($r=0.13$; $P=0.15$).

Linkage disequilibrium

There was significant linkage disequilibrium (D') between the *Bcll* (G allele) and the N363S (G allele) polymorphisms in AD patients ($P=0.03$; $D'=1.0$), which was not observed in controls ($P=0.93$; $D'=0.05$). There was a weak association for the G allele of the *Bcll* and the GAA AAG allele of the ER22/23EK polymorphism in AD patients ($P=0.07$; $D'=1.0$), but there was no linkage of the G allele of the N363S and the GAA AAG allele of the ER22/23EK in either AD patients or controls.

Clinical characteristics associated with the *Bcll* (G allele) polymorphism in healthy controls and patients

Age and gender were not different among healthy controls of different genotypes (Table 3). In healthy controls, there were ethnic differences in the distribution of the genotypes. Among white healthy controls, the most prevalent genotype was the wild type CC genotype in 44%, while among healthy Asian and black controls, the heterozygous genotype (CG allele) was observed less often than in their white and mixed ancestry counterparts. There were no ethnic differences in the distribution of the *Bcll* genotypes among patients, but there were ethnic differences in *Bcll* distribution between the patients compared with the controls.

Among healthy controls, there were no differences in TC, TG, HDLC, LDLC, NEFA, hs-CRP and the proportion with small dense LDL among those who had at least one G allele, compared with the wild type (two C alleles). In AD patients, there were no differences in the prevalence of hypertension, diabetes and use of lipid-lowering therapy. The TC, TG, HDLC, LDLC, the proportion with small dense LDL, NEFA, hs-CRP, Framingham risk and hydrocortisone dose did not differ between those who harbour at least one G allele and the wild type (two C alleles). Comparison between AD patients and healthy controls revealed that the *Bcll* association with TC, LDLC, small dense LDL, TG, HDLC, NEFA and hs-CRP did not differ. There was, however,

a trend towards a greater BMI in controls compared with the patients with respect to at least one *Bcll* G allele. Overall, the *Bcll* (G allele) had minimal effect on the clinical phenotype in both patients and healthy controls.

Clinical characteristics associated with the ER22/23EK (GAA AAG base changes) polymorphisms in healthy controls and in patients with Addison's disease

Only limited numbers of patients ($n=7$) and controls ($n=10$) with the ER22/23EK polymorphism (GAA AAG base changes) were identified (Table 4). The ages of heterozygous patients and controls 43.0 (42.0–46.5) years and 40.5 (29.0–47.0) years were similar to the ages of the wild type patients and controls 46.0 (31.0–61.0) years and 42.0 (33.0–53.0) years respectively. The BMI of heterozygous patients 29.4 (28.1–35.4) kg/m² and controls 26.3 (23.9–30.6) kg/m² was greater than that of the wild type patients 24.7 (22.1–30.0) kg/m² and controls 24.2 (22.8–28.0) kg/m² respectively and differed in both cases by more than 2 kg/m². Hypertension and diabetes occurred less frequently in the heterozygous patients than in patients exhibiting the wild type. Both the heterozygous patients and controls exhibited higher TGs than their wild type counterparts, 2.17 (1.55–3.1) mmol/l and 1.77 (1.41–1.92) mmol/l vs 1.67 (1.10–2.53) mmol/l and 1.34 (0.95–2.14) mmol/l respectively. The HDLC did not appear to be affected by the presence of the ER22/23EK polymorphism. Heterozygous patients and controls had lower LDLC than the wild types, 3.52 (0.78) mmol/l and 3.46 (0.51) mmol/l vs 4.10 (1.39) mmol/l and 3.93 (1.20) mmol/l respectively. The heterozygous patients and controls exhibited higher hs-CRP 5.0 (3.4–8.05) mg/l and 4.2 (1.1–7.2) mg/l than their wild type counterparts 2.2 (1.03–6.40) mg/l and 1.5 (0.60–2.85) mg/l respectively. Heterozygous patients received a higher dose of hydrocortisone 30.0 (25.0–30.0) mg compared with the wild type patients 20.0 (20.0–30.0) mg.

Clinical characteristics associated with the N363S (A to G base change) polymorphism in healthy control subjects and patients with AD

There were limited numbers of heterozygous patients ($n=10$) and controls ($n=7$) with the N363S polymorphism (Table 5). The heterozygous patients 31.5 (23.3–44.8) years appeared to be younger than the patients with wild type 41.0 (34.3–60.8) years, but the heterozygous controls were older 49.0 (40.5–49.3) years than the wild type controls 41.0 (33.0–53.0) years. The BMI was similar in heterozygous 25.9 (22.9–27.2) kg/m² and wild type patients 24.8 (22.1–30.4) kg/m², but in controls, the BMI was

Table 3 Analysis of the effect of the *Bcl* (C to G base change) in healthy control subjects and patients with Addison's disease.

Characteristics	Homozygous	Heterozygous	Wild type	P value		
				Controls ^a	Patients ^b	Interaction ^c
Group (<i>n</i> (freq))						
Controls	17 (0.12)	53 (0.36)	77 (0.52)			0.122
Patients	11 (0.08)	67 (0.48)	63 (0.44)			
Age (years), median (IQR)						
Controls	46.5 (26.0–54.0)	41.0 (32.0–52.5)	41.0 (34.0–53.0)	0.674	0.192	0.328
Patients	30.0 (20.5–50.5)	45.0 (47.0–59.5)	50.5 (34.0–63.8)			
Gender						
Female (freq)						
Controls	11 (0.12)	34 (0.38)	45 (0.50)	0.767	0.537	0.424
Patients	6 (0.07)	39 (0.45)	42 (0.48)			
Male (freq)						
Controls	6 (0.11)	19 (0.33)	32 (0.56)			
Patients	5 (0.09)	28 (0.52)	21 (0.39)			
BMI (kg/m ²), median (IQR)						
Controls	29.4 (24.9–31.5)	26.0 (24.3–30.1)	26.5 (20.6–31.6)	0.228	0.107	0.052
Patients	22.5 (21.5–22.6)	25.0 (21.5–29.9)	27.4 (23.5–30.6)			
Hypertensive (<i>n</i> (freq))						
Patients	0 (0)	14 (0.64)	8 (0.36)	–	0.331	–
Diabetic (<i>n</i> (freq))						
Patients	1 (0.05)	11 (0.55)	8 (0.40)	–	0.349	–
TG (mmol/l), median (IQR)						
Controls	1.28 (1.08–1.75)	1.31 (0.92–1.92)	1.42 (0.98–2.15)	0.875	0.230	0.343
Patients	1.96 (1.28–2.56)	1.82 (1.34–3.76)	1.5 (1.05–2.0)			
TC (mmol/l), mean (s.d.)						
Controls	5.92 (0.98)	5.86 (1.27)	5.67 (1.30)	0.719	0.786	0.852
Patients	5.94 (2.27)	5.65 (1.65)	5.74 (1.35)			
HDLc (mmol/l), median (IQR)						
Controls	1.15 (1.09–1.37)	1.07 (0.91–1.37)	1.06 (0.97–1.2)	0.771	0.707	0.489
Patients	0.68 (0.58–0.91)	0.7 (0.54–0.98)	0.86 (0.51–1.16)			
LDLC (mmol/l), median (IQR)						
Controls	3.93 (1.0)	3.82 (1.13)	3.95 (1.21)	0.977	0.976	0.955
Patients	4.36 (1.26)	4.02 (1.60)	4.09 (1.07)			
Small dense LDL (<i>n</i> (freq))						
Controls	0 (0)	2 (0.40)	3 (0.60)	0.506	0.264	0.990
Patients	0 (0)	12 (0.71)	5 (0.29)			
NEFA (μmol/l), median (IQR)						
Controls	622 (388–940)	444 (280–576)	467 (327–642)	0.118	0.129	0.281
Patients	349 (196–448)	226 (85–566)	417 (179–707)			
Lipid-lowering (<i>n</i> (freq))						
Patients	2 (0.11)	9 (0.47)	8 (0.42)	–	0.743	–
hs-CRP (mg/l), median (IQR)						
Controls	2.2 (0.85–1.5)	1.35 (0.60–2.28)	1.55 (0.71–3.55)	0.338	0.749	0.359
Patients	0.54 (0.19–2.1)	1.65 (0.97–3.0)	1.9 (1.14–7.4)			
Hydrocortisone (mg), median (IQR)						
Patients	25.0 (13.1–30.0)	23.8 (20.0–30.0)	20.0 (15.0–30.0)	–	0.742	–
Framingham, median (IQR)						
Patients	12.0 (5.3–22.0)	14.4 (8.0–27.4)	13.0 (4.3–21.9)	–	0.395	–
TSH (mIU/l), median (IQR)						
Patients	1.23 (0.90–2.46)	1.21 (0.50–1.91)	1.66 (0.93–2.18)	–	0.199	–

P values for the number, age, gender and ethnicity were unadjusted.

^aP value: controls comparing characteristics between *Bcl* genotypes among control subjects, adjusted for BMI, age, gender and ethnicity.

^bP value: patients comparing characteristics between *Bcl* genotypes among Addison's patients, adjusted for BMI, age, gender, ethnicity, foreign ancestry and hydrocortisone dose per metre squared.

^cP value: interaction joint comparison of characteristics between *Bcl* genotypes and between Addison's patients and healthy control subjects (interaction), adjusted for BMI, age, gender and ethnicity.

greater in heterozygotes 29.9 (26.5–31.0) kg/m² than in those harbouring the wild type 24.8 (22.1–30.4) kg/m². The TG, TC, HDLC, LDLC and proportions of small dense LDL did not appear to be affected by this polymorphism. The hydrocortisone dose appeared to be greater in heterozygous patients 25.0 (20.0–30.0) mg vs wild types 20.0 (20.0–30.0) mg.

Discussion

Our novel study examined GCR polymorphisms in patients with AD and their correlation with dose of hydrocortisone or metabolic parameters, apart from a very recent publication (32). Elevated BMI, TGs and hs-CRP but lower LDLC in healthy controls and patients

Table 4 Analysis of the effect of the presence of the ER22/23EK (GAG AGG to GAA AAG base changes) polymorphism in healthy controls and in patients with Addison's disease.

Characteristics	Heterozygous	Wild type
Group (<i>n</i> (freq))		
Controls	10 (0.07)	136 (0.93)
Patients	7 (0.05)	134 (0.95)
Age (years), median (IQR)		
Controls	40.5 (29.0–47.0)	42.0 (33.0–53.0)
Patients	43.0 (42.0–46.5)	46.0 (31.0–61.0)
Gender		
Female (freq)		
Controls	7 (0.08)	83 (0.92)
Patients	6 (0.07)	81 (0.93)
Male (freq)		
Controls	3 (0.05)	54 (0.95)
Patients	1 (0.02)	53 (0.98)
BMI (kg/m ²), median (IQR)		
Controls	26.3 (23.9–30.6)	24.2 (22.8–28.0)
Patients	29.4 (28.1–35.4)	24.7 (22.1–30.0)
Hypertensive (<i>n</i> (freq))		
Patients	0 (0)	21 (1)
Diabetic (<i>n</i> (freq))		
Patients	0 (0)	20 (1)
TG (mmol/l), median (IQR)		
Controls	1.77 (1.41–1.92)	1.34 (0.95–2.14)
Patients	2.17 (1.55–3.1)	1.67 (1.10–2.53)
TC (mmol/l), mean (s.d.)		
Controls	5.48 (0.53)	5.79 (1.30)
Patients	5.27 (0.97)	5.75 (1.57)
HDLc (mmol/l), median (IQR)		
Controls	1.11 (1.06–1.27)	1.07 (0.92–1.27)
Patients	0.72 (0.53–0.86)	0.78 (0.54–1.09)
LDLc (mmol/l), mean (s.d.)		
Controls	3.46 (0.51)	3.93 (1.20)
Patients	3.52 (0.78)	4.10 (1.39)
Small dense LDL (<i>n</i> (freq))		
Controls	0 (0)	5 (1.00)
Patients	2 (0.12)	15 (0.88)
NEFA (μmol/l), median (IQR)		
Controls	550.0 (365.0–681.0)	458.0 (328.0–642.0)
Patients	450.0 (192.0–658.0)	329.0 (139.0–654.0)
Lipid-lowering (<i>n</i> (freq))		
Patients	1 (0.05)	18 (0.95)
hs-CRP (mg/l), median (IQR)		
Controls	4.20 (1.10–7.20)	1.50 (0.62–2.85)
Patients	5.00 (3.40–8.05)	2.20 (1.03–6.3)
Hydrocortisone (mg), median (IQR)		
Patients	30.0 (25.0–30.0)	20.0 (20.0–30.0)
Framingham, median (IQR)		
Patients	9.0 (3.1–16.7)	13.3 (5.9–25.7)
TSH (mIU/l), median (IQR)		
Patients	1.10 (0.56–1.38)	1.46 (0.78–2.16)

were associated with the ER22/23EK polymorphism. Patients heterozygous for either the ER22/23EK or the N363S polymorphism appeared to require higher doses of hydrocortisone. We have also shown that AD patients demonstrated higher TG, lower HDLc concentrations, a preponderance of small dense LDL and raised hs-CRP, despite having a lower BMI than controls. These intriguing findings may account for the twofold CVD risk previously described in AD patients (2).

The Prospective Cardiovascular Münster Heart (PROCAM) study, for example, showed an association

between CVD and TG, independent of LDLc and HDLc (33). TG concentrations are influenced by multiple factors including increasing age, BMI, waist-to-hip ratio, dysglycaemia, drugs and hypothyroidism (34). AD patients may be predisposed to elevated TG independently of these aforementioned factors, possibly as hydrocortisone may increase VLDL production, thereby raising TG concentration (7, 11).

Low HDLc in AD patients could be explained by aged or improper handling of samples, but the same methods were utilised in controls. Low HDLc was found in critically ill patients with adrenal dysfunction. Apolipoprotein AI concentration is also reduced along with HDLc in the acute phase (35). Beentjes *et al.* showed that hypopituitary patients who were not supplemented with GH, and were evaluated before and after receiving hydrocortisone replacement, had decreased plasma cholesterol esterification when given hydrocortisone, and thus, HDL production may be impaired in AD in the same way. Hydrocortisone decreases LCAT activity, explaining this phenomenon (36).

AD patients had more small dense LDL particles compared with controls, which may be a further contributing factor towards the observed increase in CVD mortality. Small dense LDL particles enter arterial tissue more easily than larger LDL particles, predisposing towards atherosclerosis. Small dense LDL particles are also more susceptible to oxidation. When small dense LDL predominates, it confers a three- to sevenfold increased risk of CVD (37).

CRP is an acute-phase reactant protein and CRP concentration can increase dramatically following tissue necrosis and inflammation. In the absence of an acute-phase reaction, variations in 'baseline' CRP, detected using a highly sensitive assay, are predictive of CVD risk (38). The significance of elevated CRP needs to be investigated in long-term studies of AD patients to determine whether it translates into accelerated CVD. Precise causes of atherogenic dyslipidaemia in this cohort and whether these relate to hydrocortisone doses are unknown but it is intriguing that AD patients exhibit worse CVD risk, despite a lower BMI.

We observed significant linkage disequilibrium between the *Bcll* and N363S polymorphisms in patients but not in controls and the results of this analysis should not be over-interpreted, given the small dataset and heterogeneous groups, and the possibility of random effects. No evidence of coexistent competing GCR polymorphisms was found in the same patient.

The *Bcll* polymorphism was not associated with any metabolic alteration in our study. As GCR polymorphisms only modestly alter the phenotype, it is not surprising that the *Bcll* polymorphism did not affect the phenotype measurably, as other studies have also not demonstrated meaningful associations (39, 40, 41, 42).

The ER22/23EK polymorphism was associated with an elevated BMI in healthy controls and patients.

Table 5 Analysis of the effect of the presence of N363S (A to G base change) polymorphisms in healthy controls and in patients with Addison's disease.

Characteristics	Heterozygous	Wild type
Group (<i>n</i> (freq))		
Controls	7 (0.05)	140 (0.95)
Patients	10 (0.07)	131 (0.93)
Age (years), median (IQR)		
Controls	49.0 (40.5–49.3)	41.0 (33.0–53.0)
Patients	31.5 (23.3–44.8)	41.0 (34.3–60.8)
Gender		
Female (freq)		
Controls	3 (0.03)	87 (0.97)
Patients	5 (0.06)	82 (0.94)
Male (freq)		
Controls	4 (0.07)	53 (0.93)
Patients	5 (0.09)	49 (0.91)
BMI (kg/m ²), median (IQR)		
Controls	29.9 (26.5–31.0)	24.8 (22.1–30.4)
Patients	25.9 (22.9–27.2)	24.8 (22.1–30.4)
Hypertensive (<i>n</i> (freq))		
Patients	2 (0.09)	20 (0.91)
Diabetic (<i>n</i> (freq))		
Patients	0 (0)	20 (1.0)
TG (mmol/l), median (IQR)		
Controls	1.7 (1.47–1.79)	1.35 (0.93–2.13)
Patients	1.52 (1.16–2.06)	1.71 (1.1–2.62)
TC (mmol/l), mean (s.d.)		
Controls	6.14 (1.15)	5.75 (1.27)
Patients	5.65 (1.64)	5.73 (1.54)
HDLC (mmol/l), median (IQR)		
Controls	1.03 (0.93–1.11)	1.08 (0.93–1.27)
Patients	0.88 (0.69–0.93)	0.77 (0.51–1.08)
LDLC (mmol/l), mean (s.d.)		
Controls	3.80 (0.32)	3.90 (1.20)
Patients	4.12 (1.48)	4.08 (1.38)
Small dense LDL (<i>n</i> (freq))		
Controls	0 (0)	5 (1.0)
Patients	0 (0)	17 (1.0)
NEFA (μmol/l), median (IQR)		
Controls	351.0 (350.0–672.0)	467.0 (327.0–642.0)
Patients	299.0 (205.0–486.0)	341.0 (131.0–692.0)
Lipid lowering (<i>n</i> (freq))		
Patients	2 (0.11)	17 (0.89)
hs-CRP (mg/l), median (IQR)		
Controls	2.4 (1.95–3.9)	1.5 (0.62–2.9)
Patients	1.12 (0.53–2.73)	2.5 (1.5–6.9)
Hydrocortisone (mg), median (IQR)		
Patients	25.0 (20.0–30.0)	20.0 (20.0–30.0)
Framingham, median (IQR)		
Patients	8.7 (7.4–26.2)	13.7 (3.5–22.3)
TSH (mIU/l), median (IQR)		
Patients	1.63 (0.94–1.84)	1.38 (0.76–2.15)

Both the heterozygous patients and controls respectively exhibited higher TGs and hs-CRP than their wild type counterparts. We observed reduced LDLC with this polymorphism in patients and healthy controls. Heterozygous patients appeared to require higher doses of hydrocortisone, which could be in accordance with the expected effect (glucocorticoid resistance) of this polymorphism. The greatest impact of this polymorphism was on BMI among both the healthy controls and the AD patients. If this were a true association,

we would have expected the GCR gene locus to be strongly linked to obesity in genome-wide association studies (GWAS), as we observed a BMI difference of 2 kg/m². GWAS for obesity located SNPs on chromosomes 16, but not 5, and were most likely to affect BMI, hip circumference and mass (43). It was expected that the ER22/23EK polymorphism may result in a reduced or unchanged BMI, making our findings counter-intuitive. However, the observed increase in BMI in relation to the ER22/23EK polymorphism may also reflect an increase in the muscle mass, as has been previously reported (44). We did not measure body composition in this study and therefore cannot determine whether the raised BMI reflects an increase in muscle or fat mass. The ER22/23EK polymorphism was associated with a reduction in LDLC in healthy controls of 0.47 and 0.58 mmol/l in AD patients compared with 0.80 mmol/l observed by van Rossum *et al.* (21). Greater reductions in LDLC may be due to the age difference (average age of 40.5 years for healthy control subjects vs 69.2 years in that study) and discrepant mean LDL levels (3.46 mmol/l in the healthy controls vs 4.31 mmol/l) (21). LDL increases with age, and a higher baseline LDL may be modified to a greater degree by the ER22/23EK polymorphism (45). Beneficial effects on LDLC are not universal and one study confirmed benefit by trend, but failed to reach statistical significance (21, 44, 46, 47). The ER22/23EK polymorphism was rare and it is important to recall that we present only descriptive statistics.

The N363S polymorphism did not appear to alter clinical traits substantially apart from an increase in BMI in heterozygous controls, compared with controls exhibiting the wild type. Additionally, we noted a slightly greater hydrocortisone dose in patients with this polymorphism, compared with the wild type.

We hypothesised that patients harbouring a sensitising GCR polymorphism may require lower doses of hydrocortisone, whereas those inducing glucocorticoid resistance may require higher doses, due to persistent symptoms of glucocorticoid insufficiency (48). Szczepankiewicz *et al.* (49) by contrast showed no association between any of the GCR polymorphisms and the need for increased doses of glucocorticoids for asthma. We found very small increases in dose requirements in association with the ER22/23EK, and N363S polymorphisms and AD. However, doses were prescribed on an empiric basis, and symptoms of relative glucocorticoid deficiency are difficult to assess objectively. Moreover, doses were not adjusted by uniform criteria.

The 9β polymorphism of the GCR gene was associated with an increased risk of CVD, especially in an elderly subgroup and homozygotes had increased intima media thickness with nearly threefold increased risk of CVD (50); therefore, studying this polymorphism may yield interesting data in our cohort.

Limitations

The rarity of AD limited the size of the study with attendant limitations in the power to detect genotype–phenotype associations. The ER22/23EK and the N363S polymorphisms were rare and the resultant groups were too small for statistically meaningful analysis and thus only descriptive statistics were presented. Type I errors may occur with multiple testing and the true effect should ideally be determined in a homogenous population (51). It is impossible to rule out a false-positive result, whether one does single or multiple tests. Bonferroni's correction involves decreasing the critical *P* value, reducing the probability of a type I error, but the probability of missing a true association is increased. Most 'correction for multiple testing' approaches are based on an assumption of statistically independent tests. Bonferroni corrections are deemed to be inappropriate in a genetic study such as this, as these SNPs are located very close together on the same gene in linkage disequilibrium with each other, so it is expected to obtain similar associations with all outcomes. There is no consensus on the appropriate *P* value for testing associations with multiple outcomes with SNPs in linkage disequilibrium, on a single group of individuals. There were missing data and only non-fasted samples were collected. Patients received hydrocortisone at multiple centres and differing dosing regimens could have skewed our data. The optimal study design would have been to study AD patients receiving standardised hydrocortisone doses. Nevertheless, the majority of patients were taking 0.2–0.4 mg/kg hydrocortisone, to some degree mitigating the lack of a standard hydrocortisone dose. It is conceivable that the dosage could have been adjusted based on both subjective and objective clinical parameters and as such may have induced a degree of bias. We did not take into account the frequency and magnitude of stress dosages before 3 months, which could have had an impact on the body composition and metabolic outcomes in AD. South Africa is an ethnically and genetically diverse society, in which the true effects of a polymorphism may be more difficult to demonstrate. Overall, results should be interpreted cautiously and no specific conclusions should be drawn until corroborated.

Conclusion

We observed an increase in BMI in healthy controls and patients harbouring the ER22/23EK polymorphism. However, this study was limited by small numbers of patients with this polymorphism and further studies of larger cohorts and analysis of body composition may clarify the effect of this polymorphism on anthropometric measures. Individual hydrocortisone requirements may be influenced by many factors including differences in hydrocortisone absorption and

metabolism. GCR polymorphisms may also alter hydrocortisone requirements, but we were unable to show a definite effect in this study. This is likely because of the relatively small size of our study, the rarity of some of the polymorphisms studied, the multiplicity of factors influencing hydrocortisone dose and the non-standardised way in which hydrocortisone was dosed in this study. Our study did, however, demonstrate that patients with AD often have multiple cardiovascular risk factors and that attention to these risk factors should be an integral element in the care of patients with AD.

Declaration of interest

G Johannsson has had equity in DuoCort Pharma who developed a modified release formulation and related patents who are currently owned by Viropharma. He acts as a consultant for Viropharma. The other authors declare that they have no conflict of interest.

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Author contribution statement

All the authors contributed substantially to the study design, research and preparation of the manuscript.

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