# Role of glucocorticoids in certain endocrine diseases

Ph.D. thesis

*Belema Boyle, MD*

*Semmelweis University*
*School of Ph.D. studies*

Supervisor: Dr. Csaba Balázs, MD, Ph.D, D.sc.

Thesis reviewers: Dr. Károly Cseh, MD, Ph.D, D.sc
Dr. Gábor Kovács, MD, Ph.D.

Final Exam Committee:
Head: Dr. Ilona Kovalszky, MD, Ph.D, D.sc.
Members: Dr. István Takács, MD, Ph.D.
Dr. László Kovács, MD, Ph.D.

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## I. Introduction

Glucocorticoids have various effects on physiological processes. Besides regulating carbohydrate-, protein- and lipid metabolism, they participate in regulation of immune processes, of cardio-vascular system and behavior. By their suppressive effects on immune and inflammatory reactions glucocorticoids are widely used as anti-inflammatory pharmacological agents.

Glucocorticoids exert their effects through a ligand-activated transcription factor- the glucocorticoid receptor (GR). The unliganded GR resides in the cytoplasm and upon ligand binding translocates into the nucleus and binds to DNA at the specific sequence the glucocorticoid responsive elements (GREs) and acts as a transcripition factor.

The cellular response and sensitivity to glucocorticoids vary between individuals, tissues and cell-types. Several genetic variants of the *GR* have been described previously. During my PhD study I examined those variants, which have been proven to modify the GR sensitivity.

Graves' orbitophaty (GO) is the most frequent extrathyroidal manifestation of Graves' disease. The precise nature of the autoimmune process and the source of the offending antibodies present in this disease are still poorly understood. This chronic progressive disease is characterized by enlargement of the extraocular muscle due to infiltration of lymphocytes and accumulation of glycosaminoglycan (GAG), resulting in clinical manifestations: periorbital oedema, proptosis, diplopia and optical nerve compression. The activated lymphocytes have been shown to secrete several cytokines, which induce and sustain autoimmune processes in these tissues.

I aimed to explore the potential role of the *GR* gene polymorphisms in the pathogenesis and clinical course of GO.

Several isoforms of the GR have been described, which expression level vary- under partly known and partly undiscovered circumstances. These protein variants may influence the biological responsiveness to glucocorticoids. Among these variants, two isoforms of the human GR; the GR α and the GR β seem to be the most relevant.

The pathogenesis of hormonally inactive adrenocortical adenomas is still poorly understood. These tumors present specific clinico-pathological appearance; the patients often suffer from metabolic aberrations. Some data – i.e. bilateral involvement is 9-15 % among these patients- suggest that in their pathogenesis systemic or genetic factors may play a role. Metabolic disturbances as type II diabetes mellitus, hypertension, obesity and mild disturbances of the hypothalamus-pituitary- adrenal axis are often seen in these patients and are similar to those observed in patients with mutations of *GR*.

Our research group previously showed that the N363S polymorphism of the *GR* gene is overrepresented in patients with bilateral tumors compared with healthy controls or patients with unilateral, hormonally inactive adrenal adenomas. On the other hand, GR has been detected in healthy adrenocortical tissues but the expression and pathological relevance of the two most abundant isoforms; the GR α and GR β isoform have not been studied yet. Therefore, in the second part of my study I determined and compared the expression levels of GR α and GR β isoforms on mRNAs and protein level in normal human adrenocortical tissues, non-functioning adrenocortical adenomas (NFA) and cortisol-producing adrenocortical adenomas (CPA). I evaluated whether there might be a potential correlation between expression level of isoforms and clinico-pathological parameters observed in these patients.

## II. Objectives

(1) To identify using in silico approaches *GR* gene polymorphisms associated with altered glucocorticoid sensitivity.

(2) Out of the four selected *GR* gene polymorphisms allelic frequencies of BclI, N363S and ER22/23EK polymorphisms have been already determined in Hungarian population, but we had no data about allelic frequency of the A3669G polymorphism in healthy Hungarian population. Therefore, my next aim was to determine the allelic frequency of the A3669G polymorphism in healthy Hungarian controls and compare our result to those obtained from international databases and literature.

(3) I compared the allelic frequencies of the BclI and N363S (associated with increased sensitivity to glucocorticoids) and ER22/23EK and A3669G polymorphisms (associated wit relative resistance against glucocorticoids) in GO patients to those detected in Hungarian healthy controls.

(4) I aimed to asses whether the presence of *GR* polymorphisms correlate with the development or severity of Graves' orbitophaty.

(5) I analyzed the genotype and phenotype correlations in patients with GO and *GR* polymorphisms.

In the second part of my study I evaluated the expression of GR isoforms in normal human adrenocortical tissues, hormonally inactive benign adrenocortical adenomas and cortisol-producing benign adrenocortical adenomas in order to demonstrate:

(6) that GR, GR α and GR β isoforms are present in normal human adrenocortical tissues, in hormonally inactive adrenocortical adenoma and in

cortisol-producing adrenocortical adenoma tissues using isoform-specific antibodies.

(7) that the expression levels of GR α and β isoforms on mRNA level is different in normal human adrenocortical tissues than in pathological adrenal tissues

(8) Using immunohistochemistry, the difference observed on mRNA level is also present at protein level.

## III. Patients and methods

### III.1. Patients with Graves' orbitopathy

Unrelated Hungarian patients with GO were recruited from the Department of Ophthalmology, National Institute of Neurosurgery and the Endocrine Centre of Polyclinic of the Hospitaler Brothers of St. John of God in Budapest. GO was diagnosed on clinical grounds based on the presence of typical clinical features in the context of GD. Ophthalmopathy was classified according to the recommendation by the American Thyroid Association (ATA) Committee. A total of 95 consecutive patients with GO (18 men and 77 women; age, mean (SD), 47.3 (13.1) years) referred for endocrine evaluation were enrolled. The control group included 160 healthy, unrelated Hungarian volunteers (49 men, 111 women; age 52.7 (14.7) years) without a family history of GD or other autoimmune diseases. The research followed the Declaration of Helsinki. Written informed consent was obtained after the nature and possible consequences were explained, and the research was approved by the institutional ethical committee. Patients were divided into two groups on the basis of the severity of GO; the first group (25 patients) had non-infiltrative ophthalmopathy (ATA I) or only soft tissue involvement (ATA II), whereas the

second group (70 patients) showed ophthalmopathy defined as either stage ATA III or more severe (infiltrative ophthalmopathy)

### III.2. Adrenocortical tissues

#### III.2.1. Adrenocortical tumors and normal human adrenocortical tissues

Adrenocortical tumor tissues were obtained from 25 patients undergoing adrenalectomy, including 19 patients with NFA (15 females, 4 males, median age at the time of diagnosis: 50.4±10.3 years) and 6 patients with Cushing's syndrome due to CPA (5 females and one male, median age at the time of diagnosis: 49±9.3 years). The mean diameter of NFA was 39.2±3.6mm (mean±SE) and the mean diameter of CPA was 51.6±11.3 (mean±SE). Normal adrenal tissues were collected from surgical specimens of patients subjected to nephrectomy for kidney tumors. All patients signed a written informed consent, and the study was approved by the local Ethical Committee of Semmelweis University. All patients with adrenal tumors underwent a detailed clinical, hormonal and radiological evaluation. In all cases with adrenal tumors routine pathologic examination revealed benign adrenocortical adenomas.

Immediately after removal, normal adrenals were dissected and cortical tissues were separated. Adrenocortical adenoma tissues were also carefully separated from adjacent fat and fibrous tissues. These tissue samples were snap frozen in liquid nitrogen and stored at −80 ◦C until use for RNA extraction. Paraffin-embedded tissues were also prepared from the same surgical specimens and used for routine histological examination and immunohistochemistry.

### III.3.1 Molecular genetic methods

#### III.3.1.1 *In silico* examinations

The *in silico* examination were made by using the National Center for Biotechnology Information (NCBI) databases and software packages; the *GR* gene variants were screened through the whole nucleotide database. BLAST (Basic Local Alignment Search Tool version 2.1.2.) [1c, 2c, 3c] screening program was used as a searching tool. *GR* gene sequences: NCBI Accession Numbers: M60597.1, U78506.1-U78512.1, U80946.1, U80947.1, and GR mRNA sequences (GRα X03225.1, GRβ X03348.1) were used. For polymorphism analysis ”pubmed.org/SNP tool” and “Hapmap” database were used.

#### III.3.1.2DNS isolation

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. The DNA samples were stored at −80 ◦C until use.

#### III.3.1.3 Determination of the BclI, N363S and ER22/23EK polymorphisms of the *GR* gene

The BclI and N363S polymorphisms were determined by allele-specific polymerase chain reactions, as previously described by our group. The ER22/23EK polymorphism was detected by restriction fragment length analysis followed the polymerase chain reaction amplification of the corresponding region of the *GR* gene. Heterozygote and homozygote genotypes were distinguished by DNA sequencing.

### III.3.1.4 Real-time quantitative PCR

Genotypes for the A3669G polymorphism were detected using a primer-probe set purchased as a predesigned Taqman allelic discrimination assay. The assay was performed according to the manufacturer's instructions (Applied Biosystems, Applied Biosystems Group 850 Lincoln Centre Drive Foster City, CAA) on a 7500 Fast Real Time PCR System (Applied Biosystems).

## III.3.2 Functional molecular biological methods

### III.3.2.1 RNS isolation and cDNa synthesis

Total RNA was isolated from frozen tissues using RNeasy® mini kits (Qiagen Ltd., West Sussex, UK) according to the manufacturer's protocol. After quantification and the quality check, single stranded cDNA was generated from 1µg of total RNA using Applied Biosystems High Capacity Reverse cDNA Transcrip tion Kits (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions

### III.3.2.2 mRNA expression of total GR, GR α and GRβ isoforms

For detection of total GR mRNA Taqman probe and primer were used. For the detection of GRα and GRβ mRNAs, primers and probes were designed (primer pairs were synthesized by Genosys, Sigma (Oakville, Canada) and were labeled with FAM and TAMRA). The real-time (RT) quantification assay was performed according to the manufacturer's instructions on a 7500 Fast Real Time PCR System (Applied Biosystems). The results were further normalized by correcting them for the expression of housekeeping 18S RNA.

### III.3.2.3 DNA sequencing

The specificity of primers and probes were controlled by direct DNA sequencing. DNA sequencing was performed with BigDye terminator sequencing method using BigDye 3.0 reagent kit, capillary electrophoresis was performed on 310 Genetic Analyzer. Data were analysed by SeqScape software.

### III.3.2.4 Immunohistochemistry

A total of 8 paraffin-embedded adrenal tissues (2 normal adrenals, 2 cortisol-producing adenomas and 4 non-functioning adrenocortical adenomas) were immunostained for GR, GRα and GRβ. GR immunostaining was performed using N-terminal specific antibody corresponding to amino acids 150-175. Isoform specific immunostaining was performed using a rabbit polyclonal antibody specific for GRα without cross-reactivity with human GRβ and a rabbit polyclonal antibody to GRβ corresponding to amino acids 728–742 Human breast cancer tissues were used as positive controls. For normalization β-actin was used.

### III.3.2.5 Detection of GRβ in adrenal tissues by Western blot

Snap frozen normal adrenocortical tissues and cortisol-pruducing adrenal adneoma tissues were used to determine the specificity of anti-GRβ antibody. Tissue specimens were homogenized and total protein was extracted. GRβ protein was detected by immunoblotting with GRβ antibody followed by incubation with goat anti-rabbit horseradish peroxidise conjugated secondary antibody. Membranes were stripped and re-probed with mouse monoclonal anti-β-actin antibody then incubated with antimouse HRP conjugated secondary antibody.

### III.4 Statistical analyses

Statistical analyses were performed using SPSS (version 12.0 and 15.0 SPSS Inc., Chicago, IL, USA) software package. The frequencies of genotypes were compared in patients groups by $\chi 2$ and Fischer exact test. Continues variables were compared using student T probe (normal-distribution) and/or Mann-Whitney U-test (not normal distribution). Comparison between multiple sample groups was done using ANOVA and Mann–Whitney U-test. Correlation analysis between expression of GRα and GRβ was tested by Spearman test. Correlations between clinico-pathologic variables and GR isoform expression levels were calculated by Spearmen or Pearson correlation test, depending on the data distribution. P values less than 0.05 were considered significant. The Hardy-Weinberg distribution was calculated using the $p^2+2pq+q^2=1$ formula. Statistical power analysis was performed using a web-based software package (http://wwww.dssresearch.com/toolkit/spcalc/power_p2.asp) .

## IV RESULTS

### IV.1 Results of *in silico* examinations

According to our *in silico* examinations 16 *GR* gene polymorphims were detected. Eight are exonic, 2 map in the promoter region, 3 intronic and 3 are located in the non-translated region of the *GR* gene. Of these 2 exonic polymorphisms: the ER22/23EK and the N363S have been demonstrated as functionally active. Among polymorphisms located in intronic and non-translated region of the *GR* the BclI and the A3669G were proved to alter sensitivity against glucocorticoids.

### IV.2 Allelic frequency of the A3669G polymorphism in healthy Hungarian adults

The A3669G polymorphism in Hungarian population has not been studied in Hungarian population before. I genotyped 160 healthy Hungarian adults in order to determine the allele frequency of the A3669G SNP. The allelic frequency found in Hungarian population do not differ from data published in international literature and corresponded to those found in other European populations based on data of the NCBI (National Center for Biotechnology Information ) SNP database.

### IV.3 Allelic frequencies of BclI, N363S, ER22/23EK and A3669G polymorphisms in GO patients

Allelic frequencies of the BclI, N363S, ER22/23EK and A3669G polymorphisms of the GR gene did not differ significantly between patients with GO and healthy control.

### IV.4 Association of *GR* gene polymorhisms and severity of GO

Patients were divided into two groups on the basis of the severity of GO; the first group (25 patients) had non-infiltrative ophthalmopathy (ATA I) or only soft tissue involvement (ATA II), whereas the second group (70 patients) showed ophthalmopathy defined as either ATA III or more severe (infiltrative ophthalmopathy).The frequency of the polymorphic BclI allele was significantly higher in patients with ATAI–II (0.52) compared with those with ATA III or more (0.3; $p<0.05$).No significant correlation was found in allelic frequencies of N363S, ER22/23EK and A3669G polymorphisms between the two groups. In patients with GO, there were no associations between any of the

four GR gene polymorphisms and BMI, the presence of diabetes mellitus or hypertension.

### IV.5. GR mRNA expression in normal human edrenocortical tissues and in adrenocortical adenomas.

In human normal adrenocortical tissues and adrenocortical adenoma tissues mRNA expression was examined by performing real-time quantitative PCR by using N-specific GR mRNA (which charactarises GR α- and β isoforms together)-probe and GRα- and GRβ-isoform specific probes. The GR protein was detected by immunochemistry using N-terminal specific ,and α- and β isoform-specific antibodies.

#### IV.5.1. GR, GRα and GRβ mRNA expression

Performing RT-PCR with N-terminal spcific GR probes I found that GR mRNA was detectable in all of 25 adrenocortical adenoma tissues and in 6 normal human adrenocortical tissues. The amount of GR mRNA did not differ significantly in NFA compared to normal adrenocortical tissues, however significantly higher expression level was detectable in CPAs compared to normal adrenocortical tissues.

Using RT-PCR and isoform-specific probes for GRα and GRβ mRNA I demonstrated that the GRα mRNA was present in each adrenocortical tissue examined. Compared to normal adrenocortical tissues, GRα mRNA expression was 21.9 fold higher in CPA than in normal adrenal gland. GRβ mRNA was also detected in most of adrenal tissues with the exception of 3 of the 6 normal adrenocortical tissues and 2 of the 19 NFA, however, its amount in samples with detectable GRβ mRNA was about 1000 times lower than the amount of GRα mRNA found in the same adrenal tissues. This was reflected by the

number of PCR cycles required for the detection of reliable signals, which was 10 times more for GRβ than for GRα.

GRβ mRNA expression levels were significantly higher (7,12 fold change) in CPAs compared to normal adrenocortical tissues, while expression levels of GRβ did not differ significantly in NFAs compared to normal adrenocortical tissues.

Further analysis of the data indicated a significant positive correlation between GRα and GRβ mRNAs, expressed as fold increases, in both NFA and CPA. (Person correlation test $p<0.0001$ in NFA and $p<0.005$ in CPA)

### IV.5.2. Immunohistochemistry used for detection of GR, GRα and GRβ isoforms

#### IV.5.2.1. N-terminal GR immunohistochemistry

GR immunoreactivity was detected in some nuclei and cytoplasms of cells of zona fasciculata and reticularis from normal adrenal cortex. NFA cells showed focal staining in about 30% of the cells which was localized to nuclei. In CPA tissues all cells showed strong cytoplasmic and nuclear staining for GR.

#### IV.5.2.2. GRα and GRβ immunohistochemistry

GRα immunoreactivity was detected in some nuclei of cells from zona fasciculata and reticularis of normal cortex. More abundant staining was observed, in nearly all nuclei of NFA and CPA cells. Some CPA cells showed also cytoplasmic staining for GRα.

Neither nuclear nor cytoplasmic immunostaining for GRβ was found in cells of the normal adrenal cortex and in NFA cells. However, CPA cells showed a strong cytoplasmic and nuclear staining for GRβ.

## V. CONCLUSIONS

GR gene polymorhisms in GO patients:

(1) According to *in silico* examinations 16 *GR* gene polymorphims were detected with potential functional activity. Of these I examined four polymorphisms in GO patients using molecular genetic methods.

(2) The allele frequency of the A3669G polymorphism in Hungarian population was 22,5% which corresponds to those found in other European populations.

(3) The allelic and carrier frequencies of the N363S, ER22/23EK, A3669G and BclI polymorphisms of the GR gene did not differ significantly between patients with GO and healthy control subjects.

(4) I found significantly higher allelic frequencies of the BclI variant in patients with mild (ATA I–II) compared to severe form of GO (ATA III or more), suggesting that this polymorphism is associated with a lower susceptibility for developing severe GO. This finding is particularly interesting, since BclI polymorphism is associated with an increased sensitivity to endogenous glucocorticoids, which in patients with GO may result in a suppression of immune and inflammatory reactions that may lead to an eye-protecting effect. If these results will be reproduced by others in a larger cohort of GO patients, it would be reasonable to introduce a routine assessment of the BclI polymorphism to predict the risk of severe GO.

(5) In patients with GO, there were no associations between any of the four GR gene polymorphisms and metabolic aberrations.

In the second part of my work I studied the expression of different glucocorticoid receptor isoforms in normal human adrenocortical tissues,

hormonally inactive benign adrenocortical adenomas and cortisol-producing benign adrenocortical adenomas.

(1) Using GR N-terminal specific primers and probes and N-terminal specific antibody against GR (which corresponds to both GRα and GRβ mRNA and protein, respectively) by using real-time quantitative PCR and immunohistochemistry I showed the presence of GR mRNA and protein in normal adrenocortical adenomas, NFA and CPA tissues.

(2) Performing real-time PCR using GRα és GRβ isoform-specific primers and probes I showed the expression of GRα mRNA in all adrenocortical tissues and the presence of GRβ in 3/6 adrenocortical tissues, in 17/19 NFA tissues and in all CPA tissues.

(3) Performing immunohistochemistry by using GRα and GRβ isoform-specific antibodies I showed that GRα protein was present in all adrenocortical tissues while GRβ protein was detected only in CPA cells.

(4) Compared to normal adrenocortical tissues, both GRα and GRβ mRNAs were significantly increased in CPA

(5) I showed that differences found on mRNA expression levels were reproducible on protein-level by using immunohistochemistry and Western-blot analysis.

(6) **Publications related directly to PhD thesis**

**Original articles:**

1. **Boyle B**., Korányi K, Patocs A, Liko I., Szappanos A., Bertalan R., Racz K., Balazs Cs.(2008) Polymorphisms of the glucocorticoid receptor gene in Graves' ophthalmopathy

**British Journal of Ophtalmology 92:131-134 IF: 2.689**

2. **Boyle B**, Patócs A, Likó I, Bertalan R, Lendvai N, Szappanos Á, Butz H, Rácz K, Balázs Cs.(2008) A glukokortikoid-receptor gén polimorfizmusok szerepe autoimmun betegségekben

**Magyar Belorvosi Archivum 61: 171-175**

3. **Boyle B**, Butz H, Liko I, Zalatnai A, Toth M, Feldman K, Horányi J, Igaz P, Racz K, Patocs A. (2010) Expression of glucocorticoid receptor isoforms in human adrenocortical adenomas

**Steroids 75: 695–700 IF:2.905**

**Publications not directly related to the PhD thesis**

**Original articles:**

1.Bertalan R, Patócs A, Balogh K, Tőke J, **Boyle B**, Tóth M, Kiss R, Varga I, Gláz E, Rácz K, Tulassay Zs.(2006) A phaeochromocytoma örökletes formáinak klinikai és genetikai szűrése.

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2. Bertalan R., Csabay L., Blasovics A., Rigó J. Jr, Varga I., Halász Z., Toldy E., **Boyle B**., Rácz K.(2007) Maternal hyperandrogenism beginning from early pregnancy and progressing until delivery does not produce virilisation of a female newborn.

**Gynecological Endocrinology 10:581-3 IF:1.359**

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**The Journal of Steroid Biochemistry and Molecular Biology 111:91-94 IF: 2.827**

5. Szappanos Á; Patócs A; Tőke J; **Boyle B**, Sereg M; Majnik J; Borgulya G; Varga Ibolya; Likó, István; Rácz, Károly; Tóth, Miklós. (2009) BclI polymorphism of the glucocorticoid receptor gene is associated with decreased bone mineral density in patients with endogenous hypercortisolism .
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6. Bertalan R, Patócs A, **Boyle B**, Rigó j Jr, Rácz K. (2009) The protective effect of the ER22/23EK polymorphism against an excessive weight gain during pregnancy.
**Gynecological Endocrinology 23:1-4. IF: 1.359**