PRIMARY GENERALIZED GLUCOCORTICOID RESISTANCE OR CHROUSOS SYNDROME

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ABSTRACT

Primary Generalized Glucocorticoid Resistance or Chrousos Syndrome is a rare endocrinologic condition, which affects almost all tissues, and is characterized by resistance of tissues to glucocorticoids. The clinical spectrum of Chrousos syndrome is broad, ranging from asymptomatic cases to severe cases of mineralocorticoid and/or androgen excess. At the molecular level, Chrousos syndrome has been associated with defects in the NR3C1 gene that encodes the human glucocorticoid receptor (hGR). We and others have applied molecular and structural biology methods to investigate the molecular mechanisms of action of the mutant hGRs. We demonstrated that the defective hGRs impair several steps of glucocorticoid signaling cascade depending on the position of the NR3C1 gene defect. In clinical practice, when Chrousos syndrome is suspected, a detailed personal and family history should be obtained, while physical examination should include an assessment for signs of mineralocorticoid and/or androgen excess. Suspected patients should then undergo a detailed endocrinologic evaluation with particular emphasis on the measurement of serum cortisol concentrations and determination of the 24-hour urinary free cortisol (UFC) excretion on 2 or 3 consecutive days. Affected subjects demonstrate resistance of the HPA axis to dexamethasone suppression, which may vary depending on the severity of the condition. The diagnosis of Chrousos syndrome is confirmed by sequencing of the coding region of the NR3C1 gene, including the intron/exon junctions. Treatment of Chrousos syndrome involves administration of high doses of mineralocorticoid-sparing synthetic glucocorticoids, which activate the mutant and/or wild-type hGRα, and suppress the endogenous secretion of ACTH in affected subjects. For complete coverage of this and related areas in Endocrinology, visit our free web-books, *www.endotext.org* and *www.thyroidmanager.org*.

GLUCOCORTICOIDS

Glucocorticoids (cortisol in humans, corticosterone in most rodents) are produced by the adrenal cortex and are secreted into the systemic circulation following activation of the hypothalamic-pituitary adrenal (HPA) axis. These cholesterol-derived hormones regulate a broad spectrum of physiologic functions essential for life, such as growth, reproduction, intermediary metabolism through catabolic actions, the cardiovascular tone, behavior and cognition, and play an important role in the maintenance of resting and stress-related homeostasis (**1-4**). In addition, glucocorticoids are widely used therapeutic compounds often prescribed in the treatment of inflammatory, autoimmune and lymphoproliferative disorders because of their potent anti-inflammatory and immunomodulatory effects (**1**).

THE GLUCOCORTICOID RECEPTOR GENE (*NR3C1*) AND PROTEIN ISOFORMS

At the molecular level, glucocorticoids signal through an intracellular protein, the glucocorticoid receptor (GR) (**3, 5, 6**). The human (h) GR is a member of the steroid/thyroid/retinoic acid nuclear receptor superfamily of transcription factor proteins and functions as a ligand-dependent transcription factor that influences the transcription rate of numerous glucocorticoid target genes in a positive or negative fashion. The hGR gene (*NR3C1*) consists of 9 exons and is located on chromosome 5q31.3. Exons 2-9 constitute the protein-coding exons, whereas exon 1 consists of untranslated sequence with the transcription start site connected to multiple promoters. Alternative splicing of the *NR3C1* gene in exon 9 generates two highly homologous receptor isoforms, the hGRα and hGRβ. These protein isoforms share 727 common amino acids, but then diverge, with hGRα having an additional 50 amino acids and hGRβ having an additional, nonhomologous 15 amino acids. The hGRα resides primarily in the cytoplasm of cells and represents the classic glucocorticoid receptor that binds natural and synthetic glucocorticoids and mediates the genomic and most of the nongenomic actions of these hormones. The hGRβ, on the other hand, does not bind glucocorticoid agonists, may or may not bind the synthetic glucocorticoid antagonist RU486, has intrinsic, hGRα-independent, gene-specific transcriptional activity, and exerts a dominant negative effect upon the transcriptional activity of hGRα (**3, 5-8**). Moreover, recent studies have shown that hGRβ is implicated in insulin signaling (**9**) and participates in the molecular pathogenetic mechanisms of glioma formation through regulation of β-catenin/T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional activity (**10, 11**).

The hGRα mRNA further expresses multiple isoforms by using at least 8 alternative amino-terminal translation initiation sites (**12**). All these hGRα isoforms are differentially distributed in the cytoplasm and/or the nucleus in the absence of ligand, have different transcriptional activity following ligand-induced activation and display distinct transactivation or transrepression patterns on global gene expression examined by cDNA microarray analyses (**12**). Therefore, these hGRα isoforms may differentially transduce the glucocorticoid signal to target tissues depending on their selective relative expression and inherent activities. Since hGRβ shares a common amino-terminal domain that contains the same translation initiation sites with the hGRα, the hGRβ variant mRNA might also be translated through the same initiation sites to a similar host of hGRβ isoforms. It is likely that differential cell-specific production and functional differences might also be present between the putative hGRβ translational isoforms.

The *NR3C1* gene has at least three different promoters, A, B and C. Promoter A can be used with three untranslated exons, 1A1, 1A2 and 1A3, that contain unique promoter fragments (**13**). Therefore, the *NR3C1* gene can produce five different transcripts from different promoters that encode the same hGR proteins. Through differential use of these promoters, the expression levels of hGR proteins may vary considerably among tissues. The splice and translational hGR isoforms expressed from different promoters appear to form up to 256 different combinations of homo- and hetero-dimers with varying transcriptional activities. The marked complexity in the transcription/translation of the *NR3C1* gene enables target tissues to differentially respond to circulating glucocorticoid concentrations and accounts for the highly stochastic nature of the glucocorticoid signaling pathway (**14**).

The hGRα protein consists of four functional domains (**5**). The N-terminal or immunogenic domain (NTD) is encoded by exon 2 and represents the largest domain of the receptor containing amino acids that undergo several post-translational modifications, as well as the activation function-1 (AF-1) domain that is used by the receptor as a molecular platform for the molecular interactions with coactivators (**5**). The DNA-binding domain (DBD) is expressed by exons 3 and 4, and lies between amino acids 420 and 480. This domain consists of the characteristic motif of two zinc fingers, which facilitates the interaction between the receptor and its target DNA sequences in the promoter regions of glucocorticoid-responsive genes (**5**). The ligand-binding domain (LBD) is located at the carboxyl-terminal fragment of the receptor and corresponds to amino acids 481 to 777. Encoded by exons 5-9, this region contains amino acids responsible for the binding of the receptor to natural and synthetic glucocorticoids, for the cytoplasmic-to-nuclear translocation following ligand-induced activation of the receptor, as well as for the transactivation and interaction of the receptor with coactivator molecules (AF-2 domain) in a ligand-dependent fashion. Finally, a hinge region lies between the DBD and LBD. This protein domain provides the appropriate structural flexibility to the receptor and allows the interaction of the latter with several different glucocorticoid-responsive genes (**5**).

GENOMIC AND NONGENOMIC hGR ACTIONS

At the target cell, the inactivated hGRα resides primarily in the cytoplasm as part of a hetero-oligomeric complex consisting of chaperone heat shock proteins (HSPs) 90, 70 and 50, immunophilins, as well as other proteins (**15**). HSP90 regulates ligand binding, as well as cytoplasmic retention of hGRα by exposing the ligand-binding site and masking the two nuclear localization sequences (NLS), NL1 and NL2, which are located adjacent to the DNA-binding domain (DBD) and in the ligand-binding domain (LBD) of the receptor, respectively. Upon ligand-induced activation, the receptor undergoes a conformational change that results in dissociation from this multiprotein complex and translocation into the nucleus (**Figure 1**) (**15, 16**). Within the nucleus, the receptor binds as a dimer to tandem glucocorticoid-response elements (GREs) in the promoter regions of target genes, and regulates their expression positively or negatively depending on GRE sequence and promoter context (**17, 18**). The GRE-bound hGRα stimulates the transcription of target genes by facilitating the formation of the transcription initiation complex, including the RNA polymerase II and its ancillary components (**19**). To initiate transcription, hGRα uses its transcriptional activation domains, AF-1 and AF-2, as surfaces to interact with nuclear receptor coactivators and chromatin-remodeling complexes. Several coactivators form a bridge between the DNA-bound hGRα and the transcription initiation complex, and facilitate the transmission of the glucocorticoid signal to the RNA polymerase II (**20-22**). These include: (1) The p300 and the homologous cAMP-responsive element-binding protein (CREB)-binding protein (CBP), which also serve as macromolecular docking "platforms" for transcription factors from several signal transduction cascades, including nuclear receptors, CREB, AP-1, NF-κB, p53, Ras-dependent growth factor, and STATs. In view of their central position in many signal transduction cascades, the p300/CBP coactivators are also called co-integrators; (2) The p300/CBP-associated factor (p/CAF), which interacts with p300/CBP, but is also a broad transcription coactivator; and (3) The p160 family of coactivators, which preferentially interact with the steroid hormone receptors, and include the steroid receptor coactivator-1 (SRC-1), SRC-2 and SRC-3 (**20-22**). The hGRα also interacts with several other distinct chromatin modulators through its transactivation domains, such as the mating-type

switching/sucrose non-fermenting (SWI/SNF) complex and components of the vitamin D receptor-interacting protein/thyroid hormone receptor-associated protein (DRIP/TRAP) complex (**20-22**) (**Figure 1**).
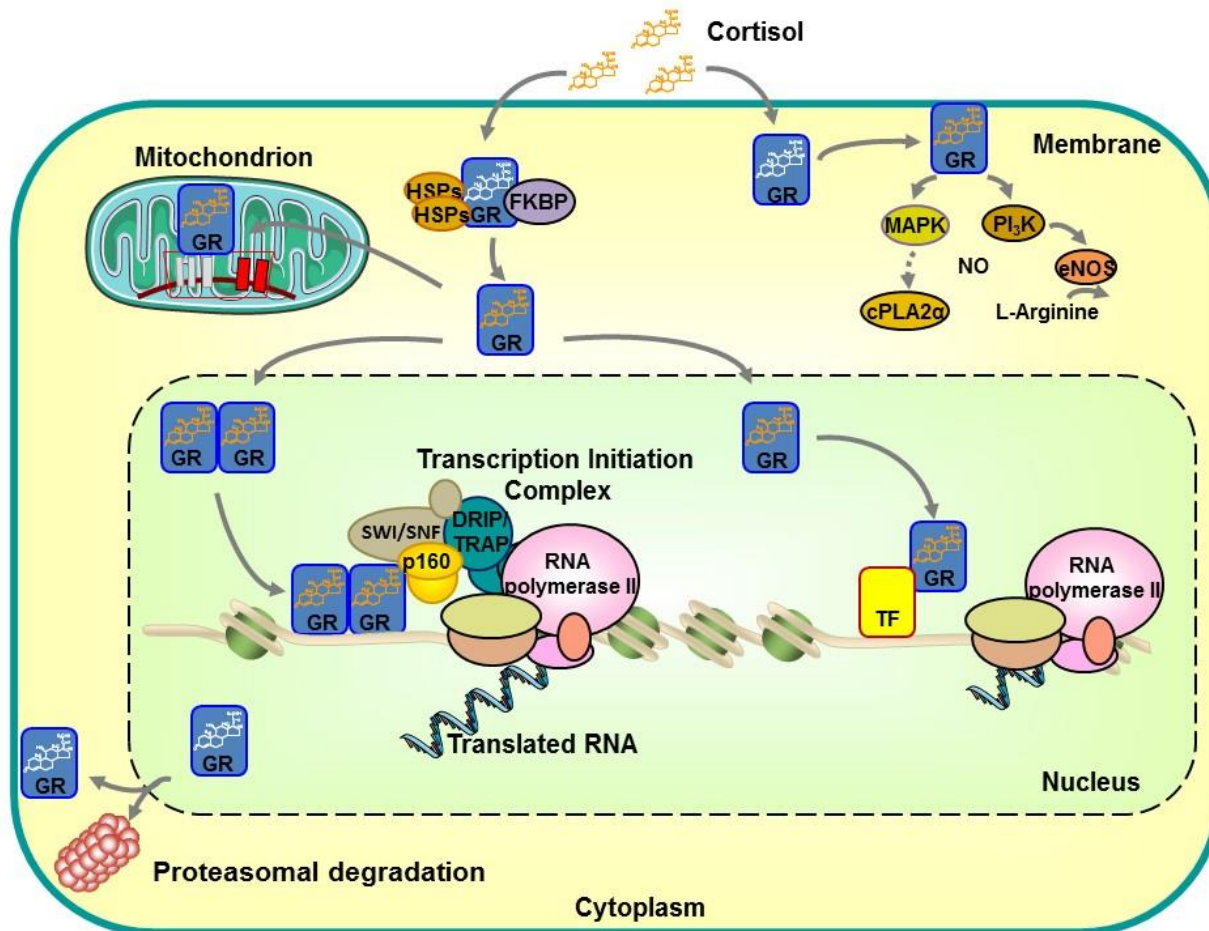

Figure 1: Genomic, nongenomic and mitochondrial glucocorticoid actions. Upon binding to the ligand, the activated hGRα dissociates from heat shock proteins (HSPs) and translocates into the nucleus, where it homodimerizes and binds to glucocorticoid response elements (GREs) in the promoter region of target genes or interacts with other transcription factors (TFs), such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB) and signal transducer and activator of transcription-5 (STAT5), ultimately modulating the transcriptional activity of respectively GRE- or TFRE-containing genes. In addition to the well-known genomic actions, most of the nongenomic glucocorticoid actions are mediated by membrane-bound GRs, which trigger the activation of kinase signaling pathways. Furthermore, accumulating evidence suggests that the ligand-bound hGRα influences the expression of mitochondrial DNA in a direct or indirect fashion. GR: glucocorticoid receptor; HSP: heat shock proteins; FKBP: immunophillins; p160: nuclear receptor coactivators p160; SWI/SNF: switching/sucrose non-fermenting complex; DRIP/TRAP: vitamin D receptor-interacting protein/thyroid hormone receptor-associated protein complex; MAPK:

mitogen-activated protein kinases; cPLA2α: cytosolic phospholipase A2 alpha; PI3K: phosphatidylinositol 3-kinase; eNOS: endothelial nitric oxide synthetase; NO: nitric oxide.

The ligand-activated hGRα can also modulate gene expression independently of binding to GREs, by interacting possibly as a monomer with other transcription factors, such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB), p53 and signal transducers and activators of transcription (STATs) (**23, 24**) (**Figure 1**). Therefore, hGRα may affect signal transduction cascades through protein-protein interactions with specific transcription factors by influencing their ability to stimulate or inhibit the transcription rates of respective target genes. This activity may be more important than the GRE-mediated one, given that mice harboring a mutant GR, which is active in terms of protein-protein interactions but inactive in terms of transactivation via DNA, survive and procreate, in contrast to mice with a deletion of the entire *NR3C1* gene that die immediately after birth from severe respiratory distress syndrome (**25**). The protein-protein interactions of GR with other transcription factors may take place on the promoters that do not contain GREs, as well as on the promoters that have both GRE(s) and responsive element(s) of transcription factors that interact with GR ("composite promoters"). Suppression of transactivation of other transcription factors through protein-protein interactions may be particularly important in suppression of immune function and inflammation by glucocorticoids (**25, 26**). Most of the effects of glucocorticoids on the immune system may be mediated by the interaction between GR and NF-κB, AP-1 and STATs (**27, 28**).

Following transcriptional activation or inhibition of glucocorticoid-responsive genes, the hGRα dissociates from the ligand and has a lower affinity for binding to GREs. The unliganded hGRα remains within the nucleus for a considerable length of time and is then exported to the cytoplasm; both within the nucleus and within the cytoplasm the hGR may be recycled and/or degraded in the proteasome (**3, 5**) (**Figure 1**).

Although the transcriptional activity of GR is primarily governed by ligand binding, accumulating evidence suggests that post-translational modifications (PTMs) play an important additional role. These include phosphorylation, ubiquitination, acetylation and sumoylation of the receptor. These covalent changes may affect receptor stability, subcellular localization, as well as the interaction between GR and other proteins (**5**).

Further to the above-described genomic actions, mounting evidence suggests that glucocorticoids also signal within seconds or minutes. These effects are termed as "nongenomic", since they do not require hGRα transcriptional activity. Although the underlying mechanisms are not fully understood, recent studies have demonstrated that most of the nongenomic glucocorticoid actions are triggered by membrane-bound GRs, which induce the activity of kinase signaling pathways, such as the mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI_3K) cascades (**29-32**) (**Figure 1**). Some representative examples of these actions are: (i) the immediate suppression of ACTH release from the anterior lobe of pituitary by glucocorticoids (**33**); (ii) the increased frequency of excitatory post-synaptic potentials in the hippocampus (**34**); (iii) the cardioprotective role of glucocorticoids through nitric oxide (NO)-mediated vasorelaxation in patients with myocardial infarction or stroke (**35**); and (iv) some immunomodulatory glucocorticoid effects via disruption of T-cell receptor signaling (**36**).

In addition to genomic and nongenomic actions, glucocorticoids exert some effects through mitochondrial hGRs, granted that many regulatory sites (D-loop) of the mitochondrial genome have functional GREs (**37**) (**Figure 1**). Several studies have shown that the ligand-activated hGRα translocates from the cytoplasm to mitochondrion and influences substantially mitochondrial gene expression (**37-39**). Moreover, many mitochondrial RNA-processing enzymes or transcription factors are expressed under the control of nuclear hGRα, suggesting a dynamic interrelation between glucocorticoids, mitochondria and the nucleus (**40**). Importantly, the mitochondrial hGRα has been early recognized as a potent therapeutic target, because of its involvement in the programmed cell death

(apoptosis) of malignant cells. Indeed, nowadays, synthetic glucocorticoids are the cornerstone of several therapeutic protocols of hematologic malignancies (**40**).

CHROUSOS SYNDROME

The internal equilibrium of all living organisms, termed *homeostasis*, is adequately achieved by the optimal effect of all homeostatic systems that occurs in the middle range of homeostatic activity. Too much or too little activity ultimately leads to dysfunction of homeostasis, termed *allostasis* or *cacostasis* (**41**). Alterations in any step of glucocorticoid signal transduction may cause impaired tissue sensitivity to glucocorticoids, which may present with clinical manifestations of *glucocorticoid resistance* or *glucocorticoid hypersensitivity*, conditions with significant morbidity (**42, 43**). One such condition that we have extensively investigated both at clinical and molecular level over the years is Primary Generalized Glucocorticoid Resistance or Chrousos syndrome (**44-56**).

Clinical Manifestations

Primary Generalized Glucocorticoid Resistance is a condition first described and elucidated by Chrousos *et al.* as a rare, familial or sporadic, genetic disorder characterized by generalized, partial, end-organ insensitivity to glucocorticoids (**47-58**). Because of the generalized glucocorticoid resistance, the glucocorticoid negative feedback inhibition at the hypothalamic and anterior pituitary levels is decreased, leading to compensatory activation of the HPA axis, inferred hypersecretion of corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) in the hypophysial portal system and increased secretion of adrenocorticotropic hormone (ACTH) in the systemic circulation (**47-58**) (**Figure 2**). The excess ACTH secretion results in adrenocortical hyperplasia and increased secretion of cortisol and adrenal steroids with mineralocorticoid [deoxycorticosterone (DOC) and corticosterone] and/or androgenic [androstenedione, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS)] activity (**47-58**) (**Figure 2**). In recognition of Professor George P. Chrousos' extensive and ground-breaking research work in this field, it has been proposed that the term "Chrousos Syndrome" is used in place of "Primary Generalized Familial or Sporadic Glucocorticoid Resistance" (**48**).
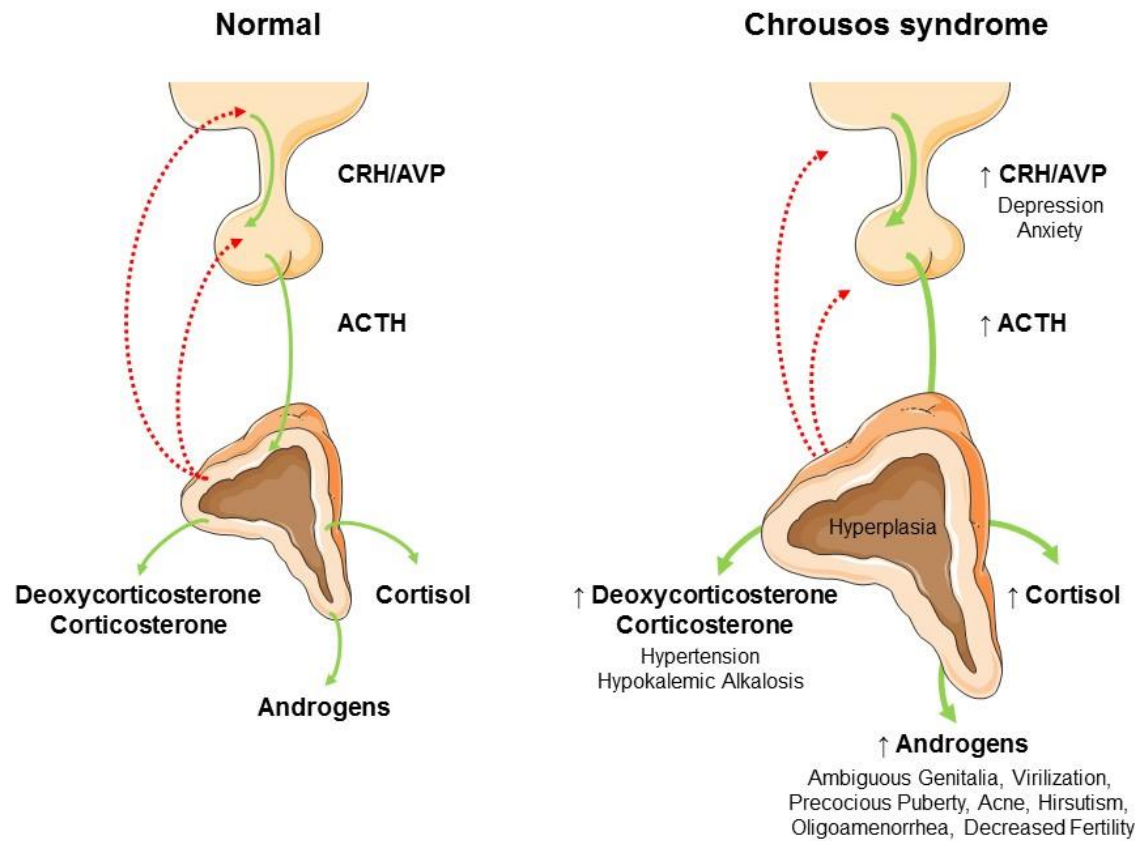
Figure 2: Pathophysiologic mechanisms and clinical manifestations of Chrousos syndrome. CRH: corticotropin-releasing hormone; AVP: arginine-vasopressin; ACTH: adrenocorticotropic hormone. Modified from Reference (**54**).

The clinical presentation of Chrousos syndrome reflects the pathophysiologic alterations described above and is mainly associated with, respectively, hypertension and/or hypokalemic alkalosis and hyperandrogenism (**47-58**) (**Table 1**) (**Figure 2**). Clinical manifestations of glucocorticoid deficiency might occur, but are rare and were only reported in a young child with hypoglycemic generalized tonic-clonic seizures during the course of a febrile illness (**59**), in a newborn baby with severe hypoglycemia, excessive fatigability with feeding, increased susceptibility to infections and concurrent growth hormone deficiency (**60**), and in several adult patients with chronic fatigue. The latter might indicate inadequate glucocorticoid target tissue compensation at the central nervous system (CNS) and/or the skeletal muscles by the increased circulating cortisol concentrations (**47-58**). Clinical manifestations of androgen excess include ambiguous genitalia in a karyotypic female at birth and gonadotropin-independent precocious puberty in children of either gender; acne, hirsutism and decreased fertility in both sexes; male-pattern hair loss, menstrual irregularities and oligo-anovulation in females; and oligospermia in males (**Table 1**). The impaired fertility in both sexes has been attributed in part to the feedback inhibition of gonadotropin secretion by the elevated androgen concentrations, while

the profound anxiety observed in some subjects is probably due to compensatory increases in hypothalamic CRH and AVP secretion. The latter might also predispose the patients to the development of an ACTH-secreting pituitary adenoma. Finally, the elevated circulating ACTH concentrations may be responsible for the observed growth of intra-testicular adrenal rests and oligospermia (**47-58**).

The clinical spectrum of Chrousos syndrome is broad, ranging from most severe to mild forms, and a number of patients may be asymptomatic, displaying biochemical alterations only (**47-58**) (**Table 1**). This variable clinical phenotype is due to variations in the tissue sensitivity of the glucocorticoid, mineralocorticoid and/or androgen receptor signaling pathways; variations in the activity of key hormone-inactivating or -activating enzymes, such as the 11β-hydroxysteroid dehydrogenase (**61**) and 5α-reductase (**62**); and other genetic or epigenetic factors, such as the presence of insulin resistance and visceral obesity (**58**).

Molecular Mechanisms

The molecular basis of Chrousos syndrome has been ascribed primarily to mutations in the *NR3C1* gene, which impair the molecular mechanisms of hGR action and decrease tissue sensitivity to glucocorticoids. The pathologic *NR3C1* gene mutations causing Chrousos syndrome that have been reported to date are shown in **Table 2** (**56, 57, 59, 60, 63-82**) and **Figure 3**. Eight-teen out of 22 of these mutations are heterozygous (4 are homozygous), while all mutations partially inactivate hGR function. Although studies of GR knock-out mice suggested that complete loss-of-function of the GR is incompatible with extrauterine life (**83**), one out of 15 of the mutations completely inactivated hGR function (**60**). The first described hGR gene mutation was a homozygotic adenine to thymine substitution at nucleotide position 1922, which resulted in substitution of aspartic acid to valine at amino acid residue 641 (**57**). *In vitro* studies showed that the mutant receptor hGRαD641V demonstrated decreased ability to transactivate glucocorticoid-responsive genes, had lower affinity for the ligand, showed delayed nuclear translocation when exposed to ligand and interacted with the glucocorticoid receptor interacting protein 1 (GRIP1) less effectively (**57**).
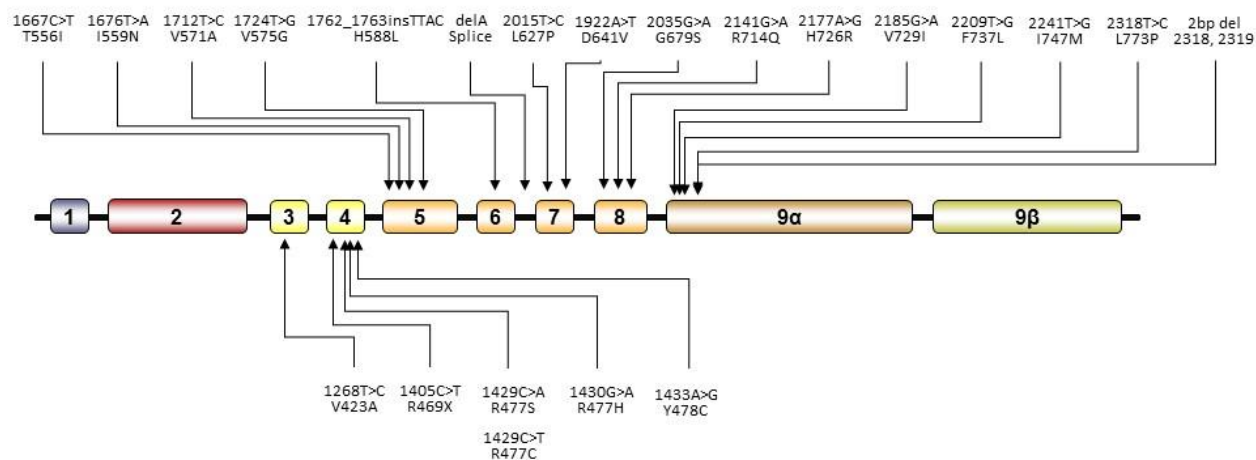
Figure 3: Location of the known mutations of the NR3C1 gene causing Chrousos syndrome. Mutations in the upper panel are located in the LBD of the receptor, while mutations in the lower panel are located in the DBD. Modified from Reference (**56**).

The molecular mechanisms through which these various natural hGR mutants affected glucocorticoid signal transduction were systematically investigated in all reported cases with the condition. These mechanisms included: i) the transcriptional activity of the mutant receptors; ii) the ability of the heterozygous mutant receptors to exert a dominant negative effect upon the wild-type receptor; iii) the concentrations and affinity of the mutant receptors for the ligand; iv) the subcellular localization of the mutant receptors and their nuclear translocation following exposure to the ligand; v) the ability of the mutant receptors to bind to GREs; vi) the interaction of the mutant receptors with the glucocorticoid receptor-interacting protein 1 (GRIP1) coactivator, which belongs to the p160 family of nuclear receptor coactivators and plays an important role in hGRα-mediated transactivation of glucocorticoid-responsive genes; vii) the motility of the mutant receptors inside the nucleus; viii) the ability of the mutant receptors to transrepress the NF-κB signaling pathway; and ix) structural biology studies (**56, 57, 59, 60, 63-82**).

The molecular defects that have been elucidated in cases with Chrousos syndrome are summarized in **Table 2**. Compared with the wild-type receptor, all mutant receptors demonstrated variable reduction in their ability to transactivate glucocorticoid-responsive genes following exposure to dexamethasone, with the most severe impairment observed in the cases of V423A, R477H, I559N, V571A, D641V, R477S and L672P mutations (**56, 57, 59, 60, 63-82**). Furthermore, the mutant receptors hGRαI559N, hGRαR714Q, hGRαF737L, hGRαI747M and hGRαL773P exerted a dominant negative effect upon the wild-type receptor, which might have contributed to manifestation of the disease at the heterozygote state (**59, 63, 67, 70, 72, 74**). All mutant receptors in which the mutations were located in the LBD of the receptor showed a variable reduction in their affinity for the ligand, with the most severe reduction observed in the cases of I559N, I747M and V571A mutations (**56, 57, 59, 60, 63-82**). The only mutant receptors that demonstrated normal affinity for the ligand were the hGRαV423A, the hGRαR477H, the hGRαR477S and the hGRαY478C in which the mutations were located at the DBD (**73, 77, 82**).

In subcellular localization and nuclear translocation studies, the pathologic mutant receptors were observed primarily in the cytoplasm of cells in the absence of ligand, except for the hGRαV729I and hGRαF737L receptors, which were localized both in the cytoplasm and the nucleus of cells. Exposure to dexamethasone induced a slow translocation of the mutant receptors into the nucleus, which ranged from 20 min (R477H) to 180 min (I559N and F737L) compared with the wild-type hGRα, which required only 12 min for complete translocation (**56, 57, 59, 60, 63-82**). These findings suggest that all hGR mutations affect the nucleocytoplasmic shuttling of the receptor, probably through impairment of the nuclear localization signal (NL)-1 and/or NL2 functions (**84**). Interestingly, the mutant receptors hGRαV423A, hGRαR477S and hGRαY478C also show delayed cytoplasmic-to-nuclear translocation upon exposure to dexamethasone, compared with the wild-type hGRα. Although these mutations are located in the DBD of the receptor, they indirectly affect the function of NL1 through R477, possibly leading to delayed cytoplasmic-to-nuclear translocation (**77, 82**).

All mutant receptors in which the mutations were located in the LBD preserved their ability to bind to DNA (**56, 57, 59, 60, 63-82**). The six mutant receptors that failed to bind to DNA were the hGRαR469X, the hGRαR477S, the hGRαR477C, the hGRαR477H and the hGRαY478C, in which the mutations were located at the C-terminal zinc finger of the DBD, and the hGRαV423A, in which the point substitution was found in the first zinc finger of the DBD of the receptor (**73, 75, 77, 82**). A major function of the C-terminal zinc finger of the DBD of hGRα is to contribute to receptor homodimerization, a prerequisite for potent receptor binding to GREs and efficient transactivation of glucocorticoid-responsive genes (**85**). The majority of the mutant receptors displayed an abnormal interaction with the GRIP1 (SRC-2) coactivator *in vitro* (**56, 57, 59, 60, 63-82**). Moreover, all mutant receptors had dynamic motility defects inside the nucleus of living cells, possibly caused by their inability to properly interact with key partner nuclear molecules of the transcription initiation complex necessary for full activation of glucocorticoid-responsive genes (**86**). Furthermore, using new-applied reporter assays, we investigated the ability of the mutant receptors hGRαV423A, hGRαV575G, hGRαH726R and hGRαT556I to transrepress the NF-κB signaling pathway. We showed that the hGRαV575G and the hGRαT556I significantly increased the transrepression of the NF-κB signaling pathway, whereas the hGRαH726R displayed decreased ability to transrepress the NF-κB signaling pathway (**77-80**).

Structural biology studies were conducted for the majority of the mutations in the *NR3C1* gene in an attempt to explain how alterations in the structure of the mutant receptors may cause generalized glucocorticoid resistance (**59, 77-80, 82, 87**). Most of the mutant receptors, in which the mutations were located in the LBD, had a defective ligand-binding pocket and/or an impaired AF-2 domain that binds to the LXXLL motifs of coactivators (**59, 78-80, 82, 87**). These

mutations resulted in loss or reduction of the electrostatic interaction between the mutant receptors and dexamethasone, ultimately leading to decreased affinity of the receptors for the ligand (**87**). The impaired interaction of the mutant receptors with coactivators was mostly due to disrupted electrostatic bonds with the non-core leucines of the LXXLL motif, as well as because of the decreased noncovalent interactions with the core leucine residues (**87**). Structural biology assays were also performed for the V423A, R477S and Y478C mutations located in the DBD (**77, 82**). In the case of V423A mutation, the substitution of valine (V) to alanine (A) destroyed the protective environment created by the hydrophobic valine for the four zinc-binding cysteines (C421, C424, C438 and C441) and permitted molecules of water to diffuse into the zinc-binding region of the receptor, therefore reducing the DNA-binding ability of the hGRαV423A (**77**). The replacement of arginine (R) by serine (S) at amino acid position 477 in the DBD of the receptor resulted in the loss of two hydrogen bonds with GREs, leading to decreased DNA binding of the mutant receptor (**82**). The substitution of tyrosine (Y) by cysteine (C) in the hGRαY478C caused a significant loss of van der Waals contacts formed between tyrosine and neighboring amino acid residues, therefore destabilizing the 3D structure of the mutated DBD (**82**).

CLINICAL EVALUATION OF THE PATIENTS

The first step in evaluating a patient with suspected Chrousos syndrome is to obtain a complete personal and family history, with particular attention to evidence suggesting hyperactivity of the HPA axis and ACTH hypersecretion-related pathology (**47-52, 54**). In addition, any evidence suggesting possible CNS dysfunction, such as headaches, visual impairment or seizures, should be noted. In female subjects, the regularity of menstrual cycles should be documented. In children and adolescents, growth and sexual maturation should be evaluated carefully, given that progressive hyperandrogenism is almost invariably associated with an increased growth velocity, an advanced bone age and changes in pubertal development (**47-52, 54**).

The physical examination should include an assessment for signs of hyperandrogenism and/or virilization, such as acne, hirsutism, pubic and axillary hair development, male-pattern hair loss and clitoromegaly. Hirsutism should be assessed using the Ferriman-Gallwey score (**88**), while pubic hair development should be classified according to Tanner (**89, 90**). Arterial blood pressure should be recorded and preferably monitored over a 24-hour period. All subjects should be screened for signs suggestive of Cushing syndrome and undergo a complete neurologic examination.

Endocrinologic Evaluation of the Patients

The concentrations of plasma ACTH, renin activity (recumbent and upright) and aldosterone, as well as those of serum cortisol, testosterone, androstenedione, DHEA and DHEAS should be recorded in the morning (**47-52, 54**). Determination of the 24-hour urinary free cortisol (UFC) excretion on 2 or 3 consecutive days is central to the diagnosis, given that patients with the condition demonstrate increased 24h UFC excretion in the absence of clinical manifestations suggestive of hypercortisolism (**47-52, 54**). The 24-hour UFC excretion may be up to 50-fold higher compared with the highest value of its normal range, while serum cortisol concentrations may be up to 7- fold higher compared with the upper normal range. Plasma ACTH concentrations may be normal or high. However, the circadian pattern of ACTH and cortisol secretion and their responsiveness to stressors are preserved, albeit at higher concentrations (**47-52, 54**).

The responsiveness of the HPA axis to exogenous glucocorticoids should also be tested with dexamethasone suppression testing (**47-52, 54**). Increasing doses of dexamethasone (0.3, 0.6, 1.0, 1.5, 2.0, 2.5, 3.0 mg) should be given orally at midnight every other day, and a serum sample should be drawn at

0800h the following morning for determination of serum cortisol and dexamethasone concentrations. Affected subjects demonstrate resistance of the HPA axis to dexamethasone suppression, which may vary depending on the severity of the condition. The concurrent measurement of serum dexamethasone concentrations is suggested in order to exclude the possibility of increased metabolic clearance or decreased absorption of this medication (**54**).

Cellular and Molecular Studies in the Patients

Thymidine incorporation assays and dexamethasone-binding assays on peripheral blood mononuclear cells in association with sequencing of the *NR3C1* gene are necessary to confirm the diagnosis and to provide genetic counseling (**47-52, 54**) (**Table 1**). In affected subjects, the thymidine incorporation assays reveal resistance to dexamethasone-induced suppression of phytohemaglutinin-stimulated thymidine incorporation, while the dexamethasone-binding assays often show decreased affinity of the hGR receptor for the ligand compared to control subjects. Sequencing of the coding region of the *NR3C1* gene, including the intron/exon junctions, will reveal insertions, deletions or mutations in most (**56, 57, 59, 60, 63-82**) but not all (**91**) cases with Chrousos syndrome. Finally, once the sequence defect is determined, its adverse effects on receptor function should be confirmed using *in vitro* mutagenesis and standardized assays that examine the ability of the mutant receptor to transactivate glucocorticoid-responsive genes.

Management of the Patients

The aim of treatment in Chrousos syndrome is to suppress the excess secretion of ACTH, thereby suppressing the increased production of adrenal steroids with mineralocorticoid and/or androgenic activity. Treatment involves administration of high doses of mineralocorticoid-sparing synthetic glucocorticoids, which activate the mutant and/or wild-type hGRα, and suppress the endogenous secretion of ACTH in affected subjects (**47-52, 54**). Adequate suppression of the HPA axis is of particular importance in cases of severe impairment of hGRα action, given that long-standing corticotroph hyperstimulation in association with decreased glucocorticoid negative feedback inhibition at the hypothalamic and pituitary levels may lead to the development of an ACTH-secreting adenoma (**63**). Long-term dexamethasone treatment should be carefully titrated according to the clinical manifestations and biochemical profile of the affected subjects (**47-52, 54**).

CONCLUSIONS AND RECOMMENDATIONS

The glucocorticoid receptor is a ubiquitously expressed intracellular, ligand-dependent transcription factor, which mediates the action of glucocorticoids and influences physiologic functions essential for life. Mutations, deletions or insertions in the *NR3C1* gene may impair one or more of the molecular mechanisms of glucocorticoid action, thereby altering tissue sensitivity to glucocorticoids. A consequent increase in the activity of the HPA axis compensates for the reduced sensitivity of peripheral tissues to glucocorticoids at the expense of ACTH hypersecretion-related pathology. The variable clinical phenotype of Chrousos syndrome, including chronic fatigue, mild hypertension and hyperandrogenism, in association with the difficulties encountered in establishing the correct diagnosis may account for the low reported prevalence of the condition, given that many cases may be unrecognized and misclassified. We recommend screening with 24h UFC excretion and sequencing of the *NR3C1* gene in patients with manifestations of mineralocorticoid and androgen excess (hypertension, hirsutism, menstrual irregularities, oligo-anovulation, impaired fertility), in whom detailed investigations fail to reveal an underlying etiology.

Although Chrousos syndrome has been associated with genetic defects in the *NR3C1* gene, some patients with clinical manifestations of this condition did not have any mutations, deletions or insertions in the gene encoding the hGR. In the era of novel technologies, we speculate that the application of whole genome/exome sequencing will delineate yet unknown molecular pathogenetic mechanisms of Chrousos syndrome, by identifying new partners that regulate the expression of the *NR3C1* gene and/or influence hGR activity.

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TABLE 1: Clinical Manifestations and Diagnostic Evaluation of Chrousos Syndrome *

Clinical Presentation

Apparently normal glucocorticoid function in most cases

       Asymptomatic

       Hypoglycemia, chronic fatigue (glucocorticoid deficiency?)

Mineralocorticoid excess

       Hypertension

       Hypokalemic alkalosis

Androgen excess

Children: Ambiguous genitalia at birth**, clitoromegaly, premature adrenarche, gonadotropin-independent precocious puberty

Females: Acne, hirsutism, male-pattern hair loss, menstrual irregularities, oligo-anovulation, hypofertility

Males: Acne, hirsutism, oligospermia, adrenal rests in the testes, hypofertility

Increased HPA axis activity (CRH/AVP and ACTH hypersecretion)

Anxiety

Adrenal rests (oligospermia)

Pituitary corticotropinoma

Diagnostic Evaluation

Absence of clinical features of Cushing syndrome

Normal or elevated plasma ACTH concentrations

Elevated serum or plasma cortisol concentrations

Increased 24-hour urinary free cortisol excretion

Normal circadian and stress-induced pattern of cortisol and ACTH secretion

Resistance of the HPA axis to dexamethasone suppression

Thymidine incorporation assays: Increased resistance to dexamethasone-induced suppression of phytohemaglutinin-stimulated thymidine incorporation compared to control subjects

Dexamethasone-binding assays: Decreased concentration or affinity of the glucocorticoid receptor for the ligand compared to control subjects

Molecular studies: Mutations/deletions of the glucocorticoid receptor; functional studies of mutant receptors

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* Modified from Reference (**48**).

** This is the only case of ambiguous genitalia documented in a child with 46,XX karyotype who also harbored a heterozygous mutation of the 21-hydroxylase gene.

TABLE 2: Mutations of the *NR3C1* Gene Causing Chrousos Syndrome

Author (Reference)	cDNA	Amino acid	Molecular Mechanisms	Genotype	Phenotype
Chrousos et al. (57) Hurley et al. (64) Charmandari et al. (71)	1922 (A→T)	641 (D→V)	Transactivation ↓ Affinity for ligand ↓ (x 3) Nuclear translocation: 22 min Abnormal interaction with GRIP1	Homozygous	Hypertension Hypokalemic alkalosis
Karl et al. (65)	4 bp deletion in exon-intron 6		hGRα number: 50% of control Inactivation of the affected allele	Heterozygous	Hirsutism Male-pattern hair-loss Menstrual irregularities
Malchoff et al. (66) Charmandari et al. (71)	2185 (G→A)	729 (V→I)	Transactivation ↓ Affinity for ligand ↓ (x 2) Nuclear translocation: 120 min Abnormal interaction with GRIP1	Homozygous	Precocious puberty Hyperandrogenism
Karl et al. (63) Kino et al. (67) Charmandari et al. (71)	1676 (T→A)	559 (I→N)	Transactivation ↓ Decrease in hGR binding sites Transdominance (+) Nuclear translocation: 180 min Abnormal interaction with GRIP1	Heterozygous	Hypertension Oligospermia Infertility
Ruiz et al.	1430 (G→A)	477	Transactivation ↓	Heterozygous	Hirsutism

(68) Charmandari et al. (73)		(R→H)	No DNA binding Nuclear translocation: 20 min		Fatigue Hypertension
Ruiz et al. (68) Charmandari et al. (73)	2035 (G→A)	679 (G→S)	Transactivation ↓ Affinity for ligand ↓ (x 2) Nuclear translocation: 30 min Abnormal interaction with GRIP1	Heterozygous	Hirsutism Fatigue Hypertension
Mendonca et al. (69) Charmandari et al. (71)	1712 (T→C)	571 (V→A)	Transactivation ↓ Affinity for ligand ↓ (x 6) Nuclear translocation: 25 min Abnormal interaction with GRIP1	Homozygous	Ambiguous genitalia Hypertension Hypokalemia Hyperandrogenism
Vottero et al. (70) Charmandari et al. (71)	2241 (T→G)	747 (I→M)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Cystic acne Hirsutism Oligo-amenorrhea
Charmandari et al. (72)	2318 (T→C)	773 (L→P)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 2.6) Nuclear translocation: 30 min Abnormal interaction with GRIP1	Heterozygous	Fatigue Anxiety Acne Hirsutism Hypertension
Charmandari et al. (74)	2209 (T→C)	737 (F→L)	Transactivation ↓ Transdominance (+)	Heterozygous	Hypertension Hypokalemia

			Affinity for ligand ↓ (x 1.5) Nuclear translocation: 180 min		
McMahon et al. (60)	2 bp deletion at nt 2318-9	773	Transactivation ↓ Affinity for ligand: absent No suppression of IL-6	Homozygous	Hypoglycemia Fatigability with feeding Hypertension
Nader et al. (59)	2141 (G→A)	714 (R→Q)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Hypoglycemia Hypokalemia Hypertension Mild clitoromegaly Advanced bone age Precocious pubarche
Bouligand et al. (75)	1405 (C→T)	469 (R→X)	Transactivation ↓ Ligand-binding sites ↓ No DNA binding No nuclear translocation	Heterozygous	Adrenal hyperplasia Hypertension Hypokalemia
Zhu Hui-juan et al. (76) Nicolaides et al. (80)	1667 (G→T)	556 (T→I)	Transactivation ↓ Transrepression ↑ Affinity for ligand ↓ Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Adrenal incidentaloma
Roberts et al. (77)	1268 (T→C)	423 (V→A)	Transactivation ↓ Affinity for ligand: Normal No DNA binding Nuclear translocation: 35 min Interaction with GRIP1: Normal	Heterozygous	Fatigue Anxiety Hypertension

Nicolaides et al. (78)	1724 (T→G)	575 (V→G)	Transactivation ↓ Transrepression ↑ Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Melanoma Asymptomatic daughters
Nicolaides et al. (79)	2177 (A→G)	726 (H→R)	Transactivation ↓ Transrepression ↓ Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Hirsutism Acne Alopecia Anxiety Fatigue Irregular menstrual cycles
Velayos et al. (81)	1429 (C→T)	477 (R→C)	Not studied yet	Heterozygous	Mild hirsutism Asymptomatic mother
Velayos et al. (81)	1762_1763insTTAC	588 (H→L*5)	Not studied yet	Heterozygous	Hirsutism Anxiety Chronic fatigue
Vitellius et al. (82)	1429 (C→A)	477 (R→S)	No Transactivation Affinity for ligand: Normal No DNA binding Nuclear translocation ↓	Heterozygous	Adrenal incidentaloma
Vitellius et al. (82)	1433 (A→G)	478 (Y→C)	Transactivation ↓ Affinity for ligand: Normal DNA binding ↓ Nuclear translocation ↓	Heterozygous	Adrenal incidentaloma
Vitellius et al. (82)	2015 (T→C)	672 (L→P)	No Transactivation No Affinity for ligand No DNA binding No Nuclear translocation	Heterozygous	Adrenal incidentaloma

DISCLOSURE STATEMENT:

The authors N.C.N., T.K., G.P.C. and E.C. have nothing to disclose.