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**Review**


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# Novel insights into the molecular mechanisms underlying generalized glucocorticoid resistance and hypersensitivity syndromes

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## ABSTRACT

**Glucocorticoids play a fundamental role in many physiologic functions and contribute substantially to the achievement of homeostasis. These pleiotropic glucocorticoid actions are mediated by a ubiquitously expressed transcription factor, the human glucocorticoid receptor (hGR), which may influence the transcription rate of numerous target genes, interact with other transcription factors, trigger the activation of several kinase pathways or modulate mitochondrial DNA expression. Any genetic defects in the *NR3C1* gene that encodes the hGR may cause Primary Generalized Glucocorticoid Resistance or Hypersensitivity Syndromes, two rare allostatic endocrinologic conditions characterized by partial impaired tissue sensitivity to glucocorticoids. However, there are patients who present with clinical manifestations suggestive of the above syndromes and do not harbor an inactivating or activating point mutation, insertion or deletion in the *NR3C1* gene. In these cases, several other factors might influence the glucocorticoid signal transduction. In this review, we discuss the numerous glucocorticoid functions and the multiple hGR isoforms, we present the genomic, nongenomic and mitochondrial glucocorticoid signaling cascade and we summarize the clinical manifestations and pathogenesis of Primary Generalized Glucocorticoid Resistance or Hypersensitivity Syndromes. Finally, we speculate that the next generation sequencing technologies will undoubtedly enable us to gain a deeper understanding of the GR “interactome”.**

**Key words:** Glucocorticoid receptor, Glucocorticoid signaling, Glucocorticoid hypersensitivity, Glucocorticoid resistance, Glucocorticoids, *NR3C1* mutations

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## GLUCOCORTICOIDS: STEROID HORMONES WITH PLEIOTROPIC FUNCTIONS

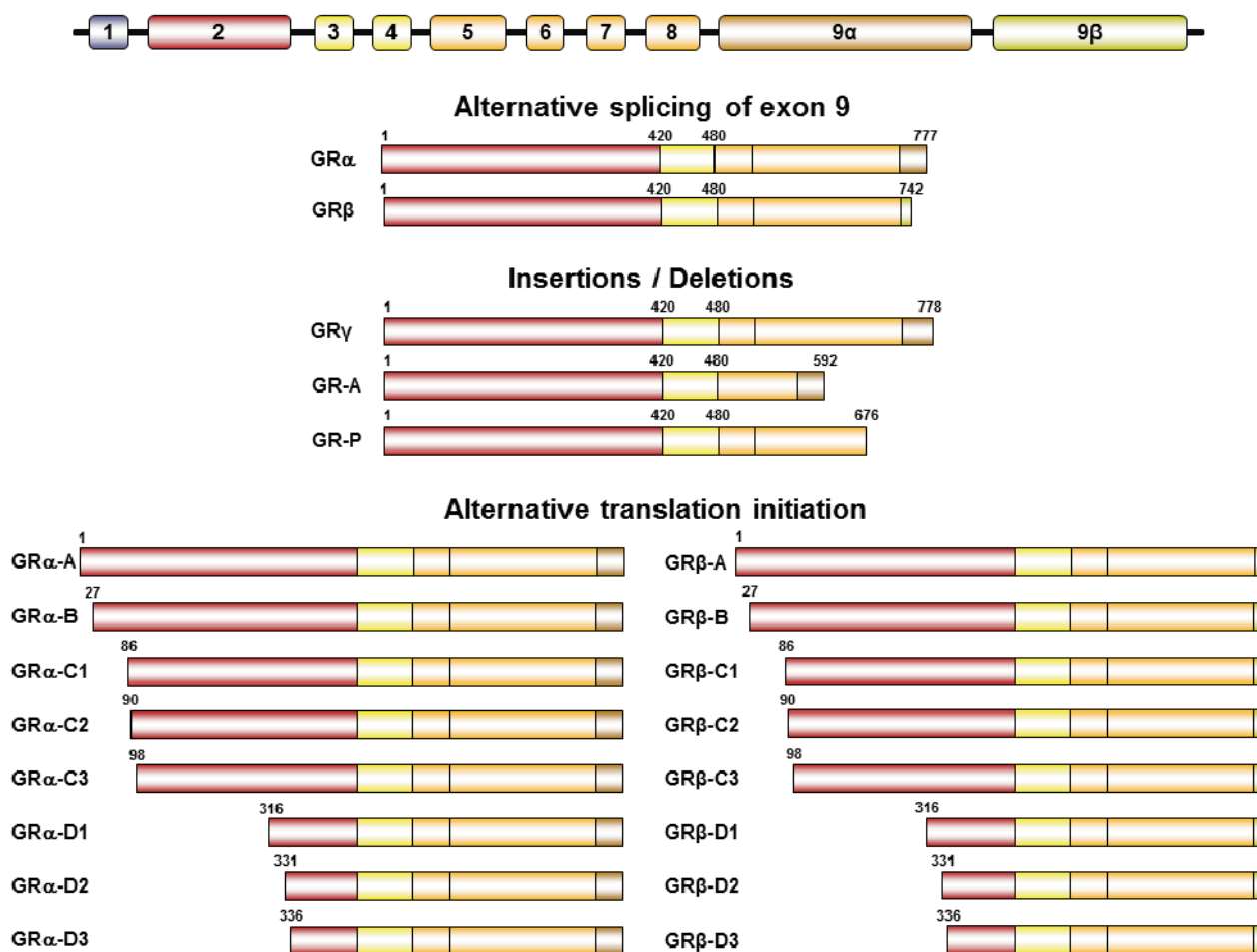
Natural glucocorticoids (cortisol in humans and corticosterone in rodents) are steroid hormones essential for life and play a crucial role in the achievement of resting and stress-related homeostasis.<sup>1-4</sup> These lipophilic molecules are derived from cholesterol through serial enzymatic reactions that lead to the production of all steroid hormones.<sup>5</sup> For many years it was believed that glucocorticoids were exclusively synthesized in endocrine cells located in the *zona fasciculata* of the adrenal cortex.<sup>4,5</sup> Recent evidence, however, points to the presence of extra-adrenal sites of glucocorticoid production, such as the intestine, thymus and skin.<sup>6</sup> Following biosynthesis, glucocorticoids are released into the systemic circulation in an ultradian, circadian and stress-related fashion.<sup>7,8</sup> The ultradian secretion of glucocorticoids occurs within a short time frame and is not influenced by neuronal input from the hypothalamic suprachiasmatic nucleus (SCN).<sup>9</sup> The circadian pattern of glucocorticoid secretion is characterized by high circulating concentrations of these hormones early in the morning and low concentrations in the evening.<sup>7</sup> Indeed, light-activated SCN neurons send projections to the paraventricular nucleus (PVN) neurons of the hypothalamus, which synthesize and release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophysial portal system.<sup>10</sup> CRH and AVP then reach the anterior lobe of the pituitary gland and trigger synergistically the production and secretion of adrenocorticotropin hormone (ACTH).<sup>1-5</sup> Finally, ACTH activates the biosynthetic pathway of glucocorticoids, while it also controls androgen and aldosterone secretion by the *zona reticularis* and *zona glomerulosa*, respectively, in the adrenal cortex.<sup>5</sup> In addition to the SCN-activated hypothalamic-pituitary-adrenal (HPA) axis, the circadian pattern of glucocorticoid release is influenced by the SCN-derived autonomic innervation of the adrenal glands as well as by circadian clocks located in the adrenal cortex.<sup>11</sup> Under acute stress, glucocorticoids are secreted in high amounts and cause a transient desynchronization of the central and peripheral clocks, which is effectively restored within a few days.<sup>8</sup> However, under chronic stress this resynchronization may not occur, leading to several metabolic, inflammatory, mood and malignant disorders.<sup>8</sup>

Glucocorticoids substantially influence myriad physiologic functions.<sup>1-4,7,8</sup> They maintain tight control over cardiovascular tone, display catabolic actions in the liver, muscle and adipose tissue and exert potent anti-inflammatory and immunosuppressive effects.<sup>1-4,7,8</sup> Moreover, glucocorticoids play a fundamental role in growth, development, reproduction, behavior and cognition. They are also involved in a very large number of cellular processes, such as proliferation, differentiation and programmed cell death (apoptosis).<sup>1-4,7,8</sup> Interestingly, a growing body of evidence suggests that glucocorticoids can cause epigenetic alterations, such as changes in the methylation status of several cytosine-guanine dinucleotides (CpG) within a number of promoter regions, by changing the expression and/or activity of enzymes involved in the methylation/demethylation process.<sup>12</sup> All these pleiotropic effects of glucocorticoids are mediated by the glucocorticoid receptor (GR), which functions as a ligand-activated transcription factor influencing the transcription rate of approximately 20% of the human genome.<sup>13,14</sup> Based on the above-discussed immunomodulating effects, synthetic glucocorticoid analogues are worldwide the cornerstone in the treatment of numerous inflammatory, autoimmune and malignant diseases.<sup>15</sup>

## GLUCOCORTICOID RECEPTOR: FROM THE *NR3C1* GENE TO PROTEIN ISOFORMS

The human GR (hGR) is a member of the steroid hormone receptor family of the nuclear receptor superfamily of transcription factors.<sup>13</sup> It is encoded by the *NR3C1* gene (chromosome 5) and consists of 10 exons. Exon 1 contains multiple tissue-specific non-coding DNA regions, whereas exons 2-9 $\alpha$ /9 $\beta$  form the protein expression region.<sup>13,16-18</sup> Through alternative splicing, insertions or deletions and via alternative translation initiation, the *NR3C1* gene finally encodes multiple protein isoforms with distinct properties in terms of localization and activity<sup>13,16-18</sup> (Figure 1).

The alternative splicing of exon 9 generates the two main receptor isoforms, termed hGR $\alpha$  and hGR $\beta$ .<sup>13,16-18</sup> These isoforms share the first 727 amino acids but display structural differences beyond this amino acid position. hGR $\alpha$  contains an additional 50 amino acids, whereas hGR $\beta$  is composed of 15 additional non-



**Figure 1.** Genomic DNA, protein isoforms and functional domains of hGR. The *NR3C1* gene consists of 10 exons. Alternative splicing of exon 9 generates the two main protein isoforms, hGR $\alpha$  and hGR $\beta$ . hGR $\gamma$ , hGR-A and hGR-P are generated by insertions or deletions in the *NR3C1* gene. The initiation of hGR $\alpha$  mRNA translation might occur from 8 alternative sites giving rise to 8 different protein isoforms. We hypothesize that the same mechanism occurs in the case of hGR $\beta$ . GR: glucocorticoid receptor.

homologous amino acids. This latter characteristic confers distinct properties on hGR $\beta$ .<sup>13,16-18</sup> Indeed, hGR $\beta$  does not bind natural or synthetic glucocorticoids; it resides in the nucleus of certain cell types, such as epithelial cells and neutrophils and exerts inhibitory effects on the transcriptional activity of the hGR $\alpha$  through well-studied molecular mechanisms.<sup>19-21</sup> However, given that studies from several groups have demonstrated that hGR $\beta$  can directly induce or repress a number of target genes,<sup>22-25</sup> while Chatzopoulou and collaborators recently revealed that GR $\beta$  does not have transcriptional activity in zebrafish,<sup>26</sup> it is evident that the specific functional role of GR $\beta$  remains to be elucidated. New evidence suggests that hGR $\beta$  has an important role in insulin

signaling and might be involved in gluconeogenesis and inflammation in mouse liver.<sup>27,28</sup> Furthermore, this isoform was found to participate in the molecular pathways of glioma formation as well as in the migration of bladder cancer cells.<sup>29-31</sup> In addition to *in vitro* and animal experiments, studies in humans have shown that hGR $\beta$  expression levels are associated with glucocorticoid resistance in several inflammatory diseases (ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, asthma), hematologic malignancies (acute lymphoblastic leukemia, chronic lymphocytic leukemia) as well as in many mood disorders (major depression, schizophrenia).<sup>18,32</sup> In contradistinction to hGR $\beta$ , the hGR $\alpha$  isoform is ubiquitously expressed in every tissue except the SCN

of the hypothalamus, is primarily localized in the cytoplasm of target cells, binds natural and synthetic glucocorticoids and influences the transcription of a large number of glucocorticoid-responsive genes in a positive or negative fashion.<sup>13,16-18</sup>

There are three additional splice GR isoforms, termed GR- $\gamma$ , GR-A and GR-P, which were found to reside in cancer cells and later in normal tissues.<sup>18</sup> GR- $\gamma$  is formed by an insertion of an arginine residue at amino acid position 452 in the ligand-binding domain (LBD) of the receptor.<sup>33</sup> This isoform is expressed in several tissues, binds glucocorticoids with similar affinity to that of the wild-type receptor, but displays 50% transcriptional activity compared to hGR $\alpha$ .<sup>34</sup> GR- $\gamma$  expression has been associated with glucocorticoid resistance in acute lymphoblastic leukemia, small cell lung carcinoma and pituitary corticotroph adenomas.<sup>34,35</sup> GR-A lacks a large fragment lying between amino acid positions 490 and 674 (exons 5-7). This splice variant is unable to bind glucocorticoids because of a defective LBD.<sup>18</sup> Finally, GR-P lacks exons 7 and 8 and does not bind glucocorticoid hormones; it is expressed in normal tissues and is over-expressed in non-Hodgkin lymphoma, acute lymphoblastic leukemia and multiple myeloma.<sup>18,36</sup> This receptor isoform influences hGR $\alpha$ -induced transcriptional activity in a cell-type specific fashion<sup>37,38</sup> (Figure 1).

More than a decade ago, Lu and Cidlowski proposed the presence of an additional cohort of GR isoforms generated by alternative initiation of GR $\alpha$  mRNA translation.<sup>39</sup> They showed that eight AUG start codons located in exon 2 of the GR transcript might give rise to eight receptor isoforms, termed GR $\alpha$ -A, -B, -C1, -C2, -C3, -D1, -D2 and -D3, with progressively shorter N-terminal domains (NTDs).<sup>39</sup> We speculate that the same mechanism might occur in GR $\beta$ , GR- $\gamma$ , GR-A and GR-P. The eight GR $\alpha$  isoforms bind to glucocorticoids with similar affinity. Following ligand-induced activation, they also have a similar capability to bind to the promoter regions of target genes.<sup>18</sup> However, these GR $\alpha$  translational variants have distinct transcription properties. Each isoform, when expressed in Jurkat T-lymphoblastic leukemia cells or U2OS osteosarcoma cells, was shown to influence the transcription of a specific set of genes.<sup>40,41</sup> Only a small percentage of target genes (less than 10%) was found to be commonly regulated

by these isoforms.<sup>40,41</sup> Not only do they have a unique transcription profile, but they also possess different subcellular localization. For example, GR $\alpha$ -D was found constitutively in the nuclear compartment, whereas GR $\alpha$ -A, GR $\alpha$ -B and GR $\alpha$ -C were seen to reside primarily in the cytoplasm and to translocate to the nucleus following ligand-induced activation.<sup>39</sup> The GR $\alpha$  translational isoforms also differ in terms of tissue expression.<sup>18</sup> GR $\alpha$ -B was found mostly in the thymus and colon, whereas GR $\alpha$ -C was highly expressed in the lung, pancreas and colon.<sup>39</sup> GR $\alpha$ -D was more abundant in the spleen and bladder.<sup>39</sup> The molecular mechanisms underlying tissue expression still remain under investigation.

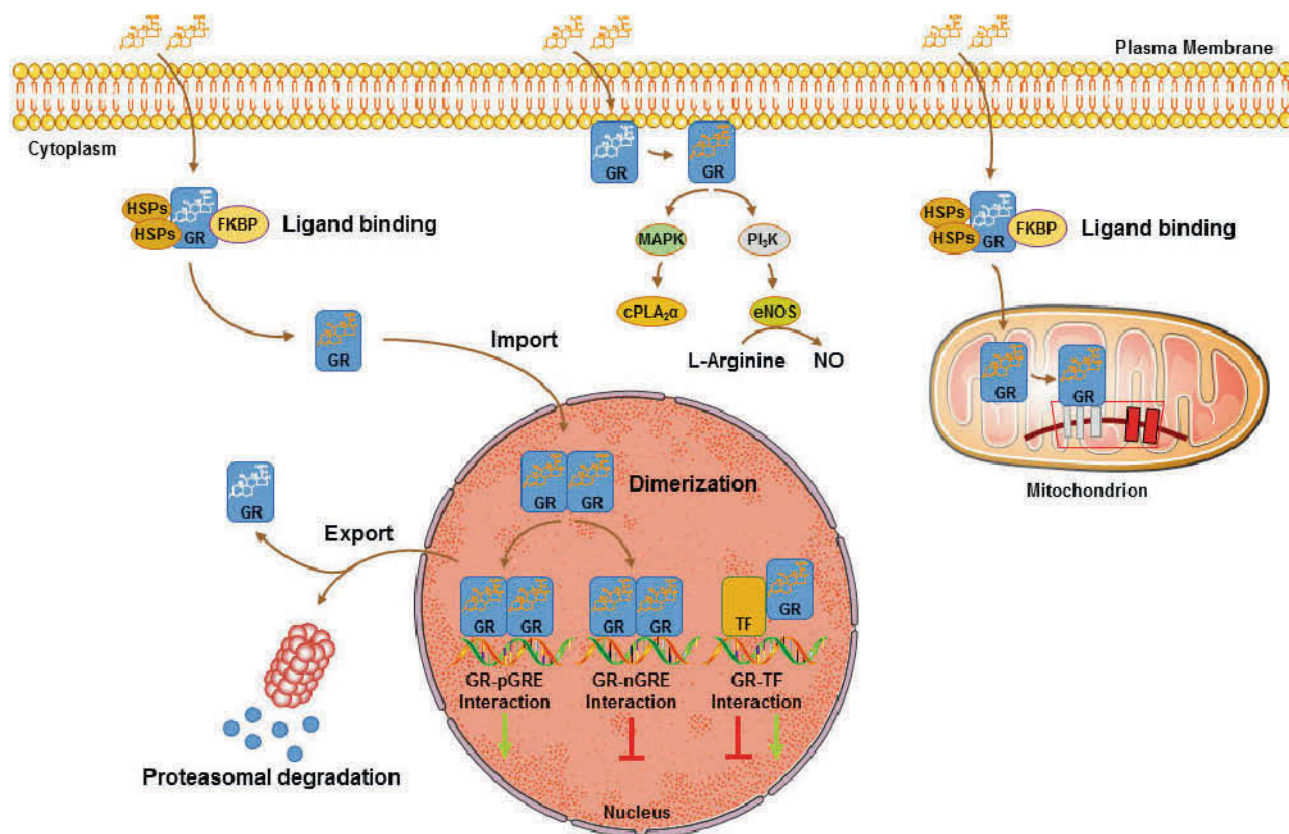
#### GENOMIC, NONGENOMIC AND MITOCHONDRIAL GR SIGNALING

At the cellular level, glucocorticoids exert their genomic, nongenomic and mitochondrial effects through activation of their cognate receptor.<sup>2,4,13,18</sup> In the absence of glucocorticoids, GR resides primarily in the cytoplasm forming a multiprotein complex that also contains heat shock proteins (HSP90, HSP70) and immunophilins (FKBP51 and FKBP52).<sup>42</sup> This complex enables GR to have a ligand-friendly conformation and masks important amino acid sequences of the receptor, nuclear localization signal 1 and 2 (NLS1 and 2), thereby preventing the nuclear translocation of the ligand-unbound receptor.<sup>43</sup> Upon ligand binding, GR undergoes an appropriate conformational change that helps it to dissociate from the multiprotein complex and then translocate to the nucleus. Within the nucleus, GR binds, as a homo- or heterodimer, to specific DNA sequences located in the promoter regions of glucocorticoid-responsive genes.<sup>2,4,13,18</sup> These sequences, termed glucocorticoid response elements (GREs), consist of two 6-nucleotide half-sites separated by three nucleotides (GGAACAnnnTGTTCT).<sup>44</sup> Following GR binding to GREs, the resultant conformational change of the GR dimer leads to the recruitment of co-activators and chromatin-remodeling complexes, which induce the activity of RNA polymerase II, thereby enabling gene transcription.<sup>18</sup> Important findings have shed light on the molecular mechanisms underlying GR-mediated gene repression.<sup>45</sup> Surjit and collaborators proposed that GR dimers might bind to

negative GREs (nGREs) and recruit corepressors, such as NCoR1 and SMRT, as well as histone deacetylases, leading to repression of several target genes.<sup>45</sup> nGRE (CTCCn<sub>0-2</sub>GGAGA) is quite different from the classic GRE in terms of nucleotide sequence and the presence of a number of nucleotides between the half-sites that range from zero to two.<sup>46</sup> In addition to GR dimer-mediated transactivation or transrepression of target genes, GR might influence gene expression independently of DNA binding. These genomic actions are mediated by protein-protein interactions of GR with several transcription factors, such as activator protein-1 (AP-1), nuclear factor- $\kappa$ B (NF- $\kappa$ B) and signal transducers and activators of transcription (STATs), thereby influencing the transcriptional activity of the latter<sup>2,4,13,18</sup> (Figure 2).

Besides glucocorticoid genomic actions, a growing body of evidence indicates that these hormones can induce some effects in a short time frame.<sup>47</sup> These

effects are referred to as “nongenomic”, occur in non-nucleated cells and do not require transcription/translation processes.<sup>47</sup> Representative examples of nongenomic glucocorticoid actions are: (i) the rapid inhibition of ACTH release from the anterior lobe of the pituitary by glucocorticoid hormones;<sup>48</sup> (ii) the immediate increased frequency of excitatory postsynaptic potentials in the hippocampus upon exposure to glucocorticoids;<sup>49</sup> (iii) the rapid reduction in blood pressure in patients with myocardial or brain ischemia following glucocorticoid administration;<sup>50</sup> and (iv) the rapid disruption of the T-cell receptor (TCR) complex, this accounting for some of the immunosuppressive glucocorticoid effects.<sup>51</sup> The molecular mechanisms underlying the nongenomic glucocorticoid effects still remain an enigma in endocrine physiology and pathophysiology. Important studies have revealed a role of a membrane-bound GR that induces the activity of several kinases, such as the mitogen-activated



**Figure 2.** Genomic, nongenomic and mitochondrial glucocorticoid signaling pathways. cPLA2 $\alpha$ : cytosolic phospholipase A2 alpha; eNOS: endothelial nitric oxide synthetase; FKBP: immunophilins; GR: glucocorticoid receptor; HSP: heat shock proteins; MAPK: mitogen-activated protein kinases; NO: nitric oxide; PI<sub>3</sub>K: phosphatidylinositol 3-kinase; TF: transcription factor.

protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI<sub>3</sub>K) pathways.<sup>52</sup> However, the nature of this membrane-anchoring GR has not yet been clarified. Although most of the steroid receptors were shown to incorporate palmitic acid, a post-translational modification termed “S-palmitoylation” that enables membrane binding of proteins and increases protein hydrophobicity,<sup>53,54</sup> we and others have demonstrated that GR $\alpha$  does not undergo S-palmitoylation, implying that other mechanisms are responsible for GR $\alpha$  membrane localization and mediate the nongenomic glucocorticoid actions<sup>55-57</sup> (Figure 2).

In addition to cytoplasm, nucleus and plasma membrane, GR was also detected in mitochondria, a key intracellular organelle with pleiotropic functions including energy production, apoptosis, calcium homeostasis, thermogenesis and stress response.<sup>58</sup> Interestingly, GREs were more than 20 years ago identified within the regulatory sites (D-loop) of the mitochondrial genome, this suggesting a genomic interrelation between mitochondria and the nucleus.<sup>59</sup> A number of studies in several tissues have observed a cytoplasmic-to-mitochondrion GR translocation or *vice versa* in response to dexamethasone, indicating that mitochondrial GR is dynamically regulated upon exposure to glucocorticoids.<sup>59-61</sup> In addition to direct GR-GRE interaction, mitochondrial gene expression is regulated indirectly by nuclear GR-GRE interactions that result in the transcription of genes encoding mitochondrial RNA-processing enzymes, nuclear respiratory factors or mitochondrial transcription factors.<sup>58</sup> From the pharmacologic point of view, synthetic glucocorticoids are currently used in the therapeutic protocols of several hematologic malignancies due to GR-mediated apoptosis of cancer cells, a mechanism that occurs in the mitochondrion<sup>58</sup> (Figure 2).

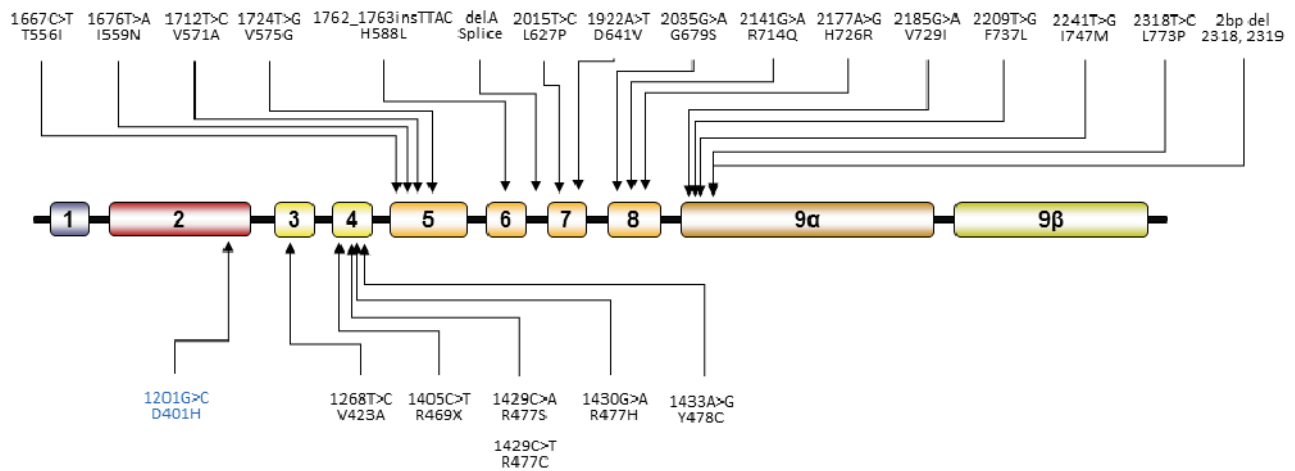
### THE GLUCOCORTICOID SIGNALING PATHWAY IN ALLOSTASIS

In humans, any alterations in glucocorticoid signal transduction may cause short- or long-term adverse effects and affect a patient’s sense of well-being and/or performance. At the molecular level, these undesired consequences may be explained by impaired tissue sensitivity to glucocorticoids that might take the form of glucocorticoid resistance or glucocorticoid

hypersensitivity, two conditions that are associated with increased morbidity.<sup>62-66</sup>

Primary Generalized Glucocorticoid Resistance (PGGR), later named “Chrousos syndrome”, is a rare endocrinologic condition which is characterized by partial tissue insensitivity to glucocorticoids.<sup>63-70</sup> Patients with this syndrome have defective glucocorticoid negative feedback loops leading to compensatory hypersecretion of both ACTH and AVP. The increased plasma ACTH concentrations cause hypertrophy of the adrenal cortex and trigger the synthesis and release of cortisol, steroid precursors with mineralocorticoid activity (deoxy-corticosterone and corticosterone) and adrenal androgens [androstenedione, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS)].<sup>63-70</sup> Depending on the functioning of these pathophysiological mechanisms, patients may have only biochemical alterations or present with symptoms and signs of mineralocorticoid excess (hypertension and/or hypokalemic alkalosis) and/or androgen excess (ambiguous genitalia at birth in karyotypic females, acne, hirsutism, precocious puberty, male-pattern hair loss and hypofertility in both sexes, oligo-amenorrhea and menstrual irregularities in women and oligospermia in men).<sup>63-70</sup> Glucocorticoid deficiency is relatively rare and has been reported in a small number of patients, such as adults with chronic fatigue,<sup>68,71,72</sup> a child presenting with hypoglycemic generalized tonic-clonic seizures that occurred during fever<sup>73</sup> and a newborn with hypoglycemia, manifesting of easy “fatigability” with feeding and growth hormone deficiency.<sup>74</sup> Some patients may also complain of profound anxiety and depression, which may be explained by the increased secretion of ACTH and AVP.<sup>70</sup> The molecular basis of Chrousos syndrome has been ascribed to inactivating point mutations, insertions or deletions in the *NR3C1* gene (Table 1, Figure 3).<sup>71,73-95</sup>

Primary Generalized Glucocorticoid Hypersensitivity (PGGH) is a rare syndrome characterized by increased tissue sensitivity to glucocorticoids mostly due to activating *NR3C1* mutations or polymorphisms.<sup>63-65</sup> Patients with this condition present with clinical manifestations of metabolic syndrome but low concentrations of serum cortisol due to compensatory hypoactivation of the HPA axis.<sup>63-65</sup> To date, only one patient has been reported with symptoms and signs of PGGH, namely visceral obesity, type 2 diabetes,



**Figure 3.** Schematic representation of the known mutations of the *NR3C1* gene causing alterations in tissue sensitivity to glucocorticoids. Mutations indicated in dark color are associated with PGGR or Chrousos syndrome, while mutation D401H, which is indicated in blue, is the only mutation that has been associated with PGGH syndrome.

**Table 1.** Mutations of the *NR3C1* gene causing Chrousos syndrome

Author (Reference)	Mutation Position		Molecular Mechanisms	Genotype	Phenotype
	cDNA	Amino acid			
Chrousos et al <sup>71</sup> Hurley et al <sup>77</sup> Charmandari et al <sup>84</sup>	1922 (A→T)	641 (D→V)	Transactivation ↓ Affinity for ligand ↓ (×3) Nuclear translocation: 22 min Abnormal interaction with GRIP1	Homozygous	Hypertension Hypokalemic alkalosis
Karl et al <sup>78</sup>	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 <sup>79</sup> Charmandari et al <sup>84</sup>	2185 (G→A)	729 (V→I)	Transactivation ↓ Affinity for ligand ↓ (×2) Nuclear translocation: 120 min Abnormal interaction with GRIP1	Homozygous	Precocious puberty Hyperandrogenism
Karl et al <sup>76</sup> Kino et al <sup>80</sup> Charmandari et al <sup>84</sup>	1676 (T→A)	559 (I→N)	Transactivation ↓ Decrease in hGR binding sites Transdominance (+) Nuclear translocation: 180 Abnormal interaction with GRIP1	Heterozygous	Hypertension Oligospermia Infertility
Ruiz et al <sup>81</sup> Charmandari et al <sup>86</sup>	1430 (G→A)	477 (R→H)	Transactivation ↓ No DNA binding Nuclear translocation: 20 min	Heterozygous	Hirsutism Fatigue Hypertension
Ruiz et al <sup>81</sup> Charmandari et al <sup>86</sup>	2035 (G→A)	679 (G→S)	Transactivation ↓ Affinity for ligand ↓ (×2) Nuclear translocation: 30 min Abnormal interaction with GRIP1	Heterozygous	Hirsutism Fatigue Hypertension

**Table 1.** (continued) Mutations of the NR3C1 gene causing Crousos syndrome

Author (Reference)	Mutation Position		Molecular Mechanisms	Genotype	Phenotype
	cDNA	Amino acid			
Mendonca et al <sup>82</sup> Charmandari et al <sup>84</sup>	1712 (T→C)	571 (V→A)	Transactivation ↓ Affinity for ligand ↓ (×6) Nuclear translocation: 25 min Abnormal interaction with GRIP1	Homozygous	Ambiguous genitalia Hypertension Hypokalemia Hyperandrogenism
Vottero et al <sup>83</sup> Charmandari et al <sup>84</sup>	2241 (T→G)	747 (I→M)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (×2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Cystic acne Hirsutism Oligo-amenorrhea
Charmandari et al <sup>85</sup>	2318 (T→C)	773 (L→P)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (×2.6) Nuclear translocation: 30 min Abnormal interaction with GRIP1	Heterozygous	Fatigue Anxiety Acne Hirsutism Hypertension
Charmandari et al <sup>87</sup>	2209 (T→C)	737 (F→L)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (×1.5) Nuclear translocation: 180 min	Heterozygous	Hypertension Hypokalemia
McMahon et al <sup>74</sup>	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 <sup>73</sup>	2141 (G→A)	714 (R→Q)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (×2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Hypoglycemia Hypokalemia Hypertension Mild clitoromegaly Advanced bone age Precocious pubarche
Bouligand et al <sup>88</sup>	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 <sup>89</sup> Nicolaidis et al <sup>93</sup>	1667 (G→T)	556 (T→I)	Transactivation ↓ Transrepression Affinity for ligand ↓ Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Adrenal incidentaloma



**Table 1.** (continued) Mutations of the NR3C1 gene causing Crousos syndrome

Author (Reference)	Mutation Position		Molecular Mechanisms	Genotype	Phenotype
	cDNA	Amino acid			
Roberts et al <sup>90</sup>	1268 (T→C)	423 (V→A)	Transactivation ↓ Affinity for ligand: N No DNA binding Nuclear translocation: 35 min Interaction with GRIP1: N	Heterozygous	Fatigue Anxiety Hypertension
Nicolaides et al <sup>91</sup>	1724 (T→G)	575 (V→G)	Transactivation ↓ Transrepression Affinity for ligand ↓ (×2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Melanoma Asymptomatic daughters
Nicolaides et al <sup>92</sup>	2177 (A→G)	726 (H→R)	Transactivation ↓ Transrepression ↓ Affinity for ligand ↓ (×2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Hirsutism, Acne, Alopecia, Anxiety, Fatigue Irregular menstrual cycles
Velayos et al <sup>94</sup>	1429 (C→T)	477 (R→C)	Not studied yet	Heterozygous	Mild hirsutism Asymptomatic mother
Velayos et al <sup>94</sup>	1762_1763insTTAC	588 (H→L*5)	Not studied yet	Heterozygous	Hirsutism, Anxiety Chronic fatigue
Vitellius et al <sup>95</sup>	1429 (C→A)	477 (R→S)	No Transactivation Affinity for ligand: N No DNA binding Nuclear translocation ↓	Heterozygous	Adrenal incidentaloma
Vitellius et al <sup>95</sup>	1433 (A→G)	478 (Y→C)	Transactivation ↓ Affinity for ligand: N DNA binding ↓ Nuclear translocation ↓	Heterozygous	Adrenal incidentaloma
Vitellius et al <sup>95</sup>	2015 (T→C)	672 (L→P)	No Transactivation No Affinity for ligand No DNA binding No Nuclear translocation	Heterozygous	Adrenal incidentaloma

hypertension and dyslipidemia.<sup>96</sup> The patient harbored a point heterozygous G → C substitution at nucleotide position 1201, which resulted in aspartic acid (D) to histidine (H) substitution at amino acid position 401 in the N-terminal domain of the receptor.<sup>96</sup> Functional characterization of hGRαD401H showed that the mutant receptor increased the expression of several

glucocorticoid-responsive genes.<sup>96</sup> In addition to activating *NR3C1* mutations, common polymorphisms associated with increased glucocorticoid sensitivity are N363S and *BclII*.<sup>18,62</sup> Moreover, only a few cases of PGGH that do not harbor a defective *NR3C1* gene have been published.<sup>97-101</sup> The etiology of these cases has not as yet been elucidated (Figure 3).

## RECENT ADVANCES IN CHROUSOS SYNDROME

We and others have systematically investigated the molecular mechanisms of action of mutant hGRs causing Chrousos syndrome by applying standard methods of molecular and structural biology (Table 1).<sup>71,73-95</sup> Recently we functionally characterized hGR $\alpha$ T556I, an *NR3C1* mutation identified in a 56-year old man with an adrenal incidentaloma.<sup>93</sup> This patient was asymptomatic and had only mildly elevated morning plasma ACTH concentrations, as well as increased 24-hour urinary free cortisol excretion (UFC).<sup>89</sup> Sequencing analysis of the *NR3C1* gene revealed a novel point C→T substitution at nucleotide position 1667, which led to a threonine to isoleucine (T→I) substitution at amino acid position 556 in the LBD of the receptor.<sup>89</sup> Our *in vitro* studies showed that the hGR $\alpha$ T556I displayed reduced ability to transactivate target genes, had decreased affinity for the ligand, demonstrated increased transrepression of the NF- $\kappa$ B responsive genes and required more time to translocate from the cytoplasm to nucleus following dexamethasone-induced activation.<sup>93</sup>

Velayos and collaborators recently described three patients with Chrousos syndrome caused by two novel point mutations in the *NR3C1* gene.<sup>94</sup> The first patient was a 12-year old girl who was referred to an endocrinologist because of high serum cortisol concentrations. She had mild hirsutism and a white stretch mark on the right thigh. Endocrinologic evaluation revealed high 24-hour urinary free cortisol excretion in two serial measurements, increased plasma ACTH and serum testosterone concentrations, as well as high salivary cortisol concentrations. The patient did not suppress serum cortisol concentrations following an overnight dexamethasone (1mg) suppression test. Interestingly, her mother was asymptomatic and had only slightly increased serum cortisol concentrations.<sup>94</sup> Both the girl and her mother harbored a novel point *NR3C1* mutation (1429C→T) in exon 4, which resulted in an arginine to cysteine (R→C) substitution at amino acid position 477 in the second zinc finger of the DBD of the receptor.<sup>94</sup> The second patient was a 41-year old woman who had had a history of hirsutism for 4 years and also presented with chronic fatigue and anxiety.<sup>94</sup> She had increased 24-hour UFC excretion and increased salivary cortisol and serum DHEA-S and testosterone concentrations. Sequencing analysis

revealed a novel insertion of four bases between nucleotides 1762 and 1763, which resulted in a substitution of four amino acids at positions 588-591 leading to a premature stop codon at amino acid position 592, and therefore to a truncated protein.<sup>94</sup>

Three novel heterozygous point mutations in the *NR3C1* gene have also been identified by Vitellius et al in patients with adrenal incidentalomas and increased circulating glucocorticoid concentrations without clinical manifestations suggestive of Cushing syndrome.<sup>95</sup> Two of these mutations, R477S and Y478C, were located in the second zinc finger of the receptor DBD, whereas the third mutation, L672P, was found in the receptor LBD.<sup>95</sup> The authors undertook a functional characterization of these three mutations. Transactivation assays showed that the mutant receptors hGR $\alpha$ R477S and hGR $\alpha$ L672P had decreased ability to transactivate glucocorticoid-responsive genes compared to the wild-type receptor (hGR $\alpha$ WT). Furthermore, the mutant receptors located in the DBD had a similar affinity for dexamethasone to that of hGR $\alpha$ WT.<sup>95</sup> In contrast, hGR $\alpha$ L672P was not able to bind to the ligand because of a defective LBD. hGR $\alpha$ R477S and hGR $\alpha$ Y478C had a marked delay in the translocation from the cytoplasm to the nucleus following ligand-binding, whereas hGR $\alpha$ L672P alone maintained its cytoplasmic localization.<sup>95</sup> As expected, the mutant receptors hGR $\alpha$ R477S and hGR $\alpha$ Y478C had reduced ability to bind to DNA compared to hGR $\alpha$ WT. Structural biology assays demonstrated that hGR $\alpha$ R477S lost two hydrogen bonds with GREs and hGR $\alpha$ Y478C displayed impaired interaction with neighboring amino acid residues, whereas hGR $\alpha$ L672P caused a conformational change of helix 8.<sup>95</sup>

## NOVEL INSIGHTS INTO PGGH

We have described a 9-year old girl with transient PGGH who presented with a long history of clinical manifestations suggestive of Cushing syndrome, including progressive weight gain, buffalo hump, moon facies, purple skin striae, acanthosis nigricans, hirsutism, myopathy, as well as decreased growth velocity.<sup>102</sup> Interestingly, her morning plasma ACTH and serum cortisol concentrations were undetectable (<1 pg/mL and 0.025 mcg/dL, respectively) and remained decreased during the 24-hour study. CRH

stimulation showed no increase in plasma ACTH and serum cortisol concentrations.<sup>102</sup> These clinical manifestations and our endocrinologic investigations pointed to a diagnosis of transient generalized glucocorticoid hypersensitivity. Sequencing analysis revealed no mutations or polymorphisms in the *NR3C1* gene.<sup>102</sup> Of note, the symptoms and signs of the patient gradually resolved over the ensuing three months. RNA sequencing was performed in peripheral blood mononuclear cells during the acute and the resolution phases of the disease and transcriptomic analysis revealed 903 differentially expressed genes. The majority of them were found to be NF- $\kappa$ B target genes, indicating that a transient post-receptor defect or a viral or bacterial protein might have enhanced hGR $\alpha$ -mediated transcriptional activity.<sup>102</sup>

#### CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Chrousos syndrome and Primary Generalized Glucocorticoid Hypersensitivity syndrome are two rare allostatic conditions which are characterized by impaired glucocorticoid signaling. The molecular basis of these conditions has been attributed to genetic defects in the *NR3C1* gene. However, some patients with clinical manifestations suggestive of these syndromes did not harbor any mutations, insertions or deletions in the *NR3C1* gene, implying that several other factors might influence the glucocorticoid signal transduction. Representative examples of such molecules are the FK506-Binding Immunophilins FKBP5. Indeed, any imbalance between the protein expression levels of FKBP51 and FKBP52 may cause glucocorticoid resistance or hypersensitivity.<sup>103</sup> In addition to hGR $\alpha$  protein partners, accumulating evidence suggests that some of the non-coding RNAs could play an important role in determining tissue sensitivity to glucocorticoids.<sup>104</sup> One of them, “Growth Arrest-Specific 5” (GAS5) long non-coding RNA, was found to suppress the expression of several glucocorticoid responsive genes through binding to the DBD of GR $\alpha$  by acting as a decoy GRE.<sup>105</sup> GAS5 was identified as an important factor influencing human response to glucocorticoids. Lucafo et al demonstrated that poor responders to glucocorticoids had increased levels of GAS5 compared to good responders.<sup>106</sup> These accumulating data evidence the fact that novel technologies,

including whole-genome sequencing, whole-exome sequencing and RNA sequencing, will undoubtedly enable us to acquire a deeper understanding of the GR “interactome”.

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