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HAL Authorization

1 **Characterization of a novel variant in the *NR3C1* gene:**
2 **differentiating glucocorticoid resistance from Cushing Syndrome**

3
4 Margaux Laulhé¹, Michal Yacobi Bach^{2,3}, Julie Perrot¹, Michal Gershinsky^{4,5}, Jérôme Fagart⁶,
5 Gabi Shefer², Larbi Amazit⁷, Peter Kamenický^{1,8}, Say Viengchareun¹, Laetitia Martinerie^{1,9,10},
6 Yona Greenman^{2,11}
7

8 ¹ Université Paris-Saclay, Inserm, Physiologie et Physiopathologie Endocrinienne, 94276 Le Kremlin-Bicêtre,
9 France;

10 ² Institute of Endocrinology, Diabetes, Metabolism and Hypertension, Tel Aviv Sourasky Medical Center, Tel Aviv,
11 Israel;

12 ³ Genetics Institute, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel;

13 ⁴ Department of Endocrinology and Diabetes, Lady Davis Carmel Medical Center and Linn Medical Center, Clalit
14 Health Services, Haifa, Israel;

15 ⁵ Ruth and Bruce Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel;

16 ⁶ CNRS UMR 7654, Ecole Polytechnique, 91120 Palaiseau, France;

17 ⁷ UMS 44/Institut Biomédical du Val de Bièvre, Université Paris-Saclay, 94276 Le Kremlin Bicêtre, France;

18 ⁸ Service d'Endocrinologie et des Maladies de la Reproduction, Hôpital Bicêtre GHU APHP Paris Saclay, 94270 Le
19 Kremlin-Bicêtre, France;

20 ⁹ Endocrinologie Pédiatrique, Centre de Référence Maladies Endocriniennes Rares de la Croissance et du
21 Développement, Hôpital Universitaire Robert-Debré GHU APHP Nord, 75019 Paris, France;

22 ¹⁰ Université Paris-Cité, Faculté de Santé, UFR de Médecine, Paris, France;

23 ¹¹ Faculty of Medical & Health Sciences, Tel Aviv University, Tel Aviv, Israel.
24

25 **Corresponding author:**

26 Yona Greenman, MD

27 Institute of Endocrinology, Diabetes, Metabolism and Hypertension

28 Tel Aviv Sourasky Medical Center

29 Tel Aviv, Israel

30 yonagr@tlvmc.gov.il

31 **ORCID ID** 0000-0003-4923-488x

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Abstract

Introduction: Primary generalized glucocorticoid resistance syndrome (GGRS) is a rare endocrine disease caused by loss-of-function variants of the *NR3C1* gene encoding the Glucocorticoid Receptor. We describe a novel heterozygous missense variant (NM_000176.3, c.1330T>G, p.Phe444Val) within the DNA Binding Domain.

Clinical case: Elevated urinary-free cortisol levels were detected in a 59-year-old male before bariatric surgery (BMI 39.9 kg/m²). Early-onset hypertension was well controlled. The low dose dexamethasone suppression test was pathologic, but ACTH and midnight salivary cortisol levels were normal. The patient was initially referred to transsphenoidal surgery for a presumed diagnosis of Cushing disease. He presented to our department at the age of 68, when the clinical diagnosis of GGRS was established.

1 **Methods:** Functional characterization of the variant was performed *ex vivo* through transient
2 transfection assays in HEK 293T cells to assess transcriptional activity and nuclear translocation.

3 **Results:** The variant showed a lack of transcriptional activity (GR_{WT}: 91.5 [80.5; 101.2] vs.
4 GR_{F444V}: 1.0 [1.0; 1.0]) despite efficient nuclear translocation in response to dexamethasone,
5 suggesting a DNA binding defect of the variant. These results are discussed in the light of
6 previously reported GGRS cases.

7 **Conclusion:** We have described a novel heterozygous mutation of the *NR3C1* gene associated
8 with primary GGRS. This case highlights the importance of raising awareness of clinical and
9 laboratory features of this rare disorder, to enable early diagnosis and avoid unnecessary and
10 potentially dangerous diagnostic and therapeutic procedures.

11

12 **Introduction**

13 The Glucocorticoid Receptor (GR) is a nuclear receptor encoded by the *NR3C1* gene¹. The GR is
14 translocated into the nucleus upon ligand binding and acts mainly through DNA binding to regulate
15 gene transcription. Depending on tissue and cell specificity, the GR may regulate up to 20% of the
16 genome, acting on glucose and lipid metabolism, osteogenesis, immunity, and cognition². More
17 than 35 variants of the *NR3C1* gene associated with a loss-of-function of the GR have been
18 reported³. These variants are responsible for primary generalized glucocorticoid resistance
19 syndrome (GGRS), also called Chrousos syndrome^{3,4}. It is characterized by biochemical
20 hypercortisolism without the characteristic clinical symptoms and signs of glucocorticoid excess
21 (Cushing syndrome) and impaired negative feedback on the Hypothalamic-Pituitary-Adrenal
22 (HPA) axis. The HPA axis controls glucocorticoid secretion by the adrenal glands under basal and
23 stress conditions. Glucocorticoids exert negative feedback on the HPA axis by binding to the GR⁵

1 in the pituitary gland, thus inhibiting adrenocorticotropin hormone (ACTH) synthesis and
2 secretion. In glucocorticoid resistance syndrome, negative feedback is altered due to the non-
3 functional GR, resulting in inappropriate normal or elevated ACTH secretion despite high cortisol
4 levels. Chronic ACTH stimulation, in addition to hypercortisolism, may lead to adrenal
5 hyperplasia and increased secretion of androgens and corticosteroids with mineralocorticoid
6 activity^{6,7}. As a result, patients may present with hypertension and hypokalemia, secondary to
7 activation of the mineralocorticoid receptor or hirsutism in women due to hyperandrogenism⁸.
8 Here we report and characterize a novel loss-of-function variant in the *NR3C1* gene associated
9 with familial GGRS, characterized by obesity, hypertension, and adenomatoid cortical
10 hyperplasia. The patient's diagnosis was delayed by ten years, during which he underwent
11 extensive evaluation for non-neoplastic hypercortisolism (NNH) and ACTH-dependent Cushing
12 syndrome. In view of the diagnostic difficulties illustrated by this case, we also conducted a
13 comprehensive review of the clinical presentations and laboratory findings of previously published
14 cases of GGRS, in order to identify distinctive features likely to improve diagnostic accuracy^{9–29}.

15

16 **Material and Methods**

17 **Clinical and biochemical parameters**

18 Clinical and laboratory data were extracted from the patient's electronic health records from the
19 Clalit Health Services and the Tel Aviv-Sourasky Medical Center. Hormonal concentrations were
20 quantified using different methods and laboratories throughout the years. The most recent
21 assessments were performed as follows: serum cortisol and plasma ACTH were determined by
22 automated electrochemiluminescent Roche cobas® e 601 ([RRID:SCR_025682](https://pubmed.ncbi.nlm.nih.gov/25682/)) analyzer (Roche

1 Diagnostics). Androstenedione and DHEAS were measured via chemiluminescent Siemens 2000
2 Immunoassay System ([RRID:SCR_020514](#)). Aldosterone levels were assessed by
3 immunochemiluminescence on the Beckman Coulter DiaSorin LIAISON XL Chemiluminescence
4 Immunoassay Analyzer ([RRID:SCR_019630](#)). Plasma renin activity and salivary cortisol were
5 analyzed using commercially available ELISA kits (DBC inc. Ref# CAN-RA-4600 and IBL,
6 Medison [RRID:SCR_001131](#), Ref # 30172091) respectively. Urinary-free cortisol concentrations
7 were determined by radioimmunoassay (Beckman Coulter, [RRID:SCR_008940](#), Ref # IM1841).

9 **Family phenotype**

10 Genetic counseling and clinical genetic testing were performed after informed consent from the
11 patient and his children. Publication of the family's clinical and molecular information was subject
12 to an informed consent waiver from the Tel Aviv Sourasky Medical Center Institutional Review
13 Board (#0039-15-TLV).

15 **Genetic testing**

16 Sequencing of the *NR3C1* gene using exome-based NGS was carried out at the Genomic Center
17 at the Tel Aviv Sourasky Medical Center, as previously described³⁰. Data analysis was performed
18 on the Franklin data analysis platform (Genoox, Tel Aviv), which enables the detection of Single
19 Nucleotide Variants and Copy Number Variants. Sequence reads were mapped to the
20 GRch37/hg19 human reference genome. Sanger sequencing was used to analyze variant
21 segregation.

1 **Cell culture**

2 Human HEK 293T cells were cultured at 37°C with 5% CO₂ in Dulbecco's Modified Eagle
3 Medium (DMEM, Life Technologies, Villebon-sur-Yvette, France) with 20 mM HEPES (Life
4 Technologies), 100 UI/mL penicillin (Life Technologies), 100 µg/ mL streptomycin (Life
5 Technologies), and supplemented with 10% of fetal bovine serum (FBS, Biowest, Nuaille, France).

7 **Site-directed mutagenesis**

8 Plasmid pcDNA3-hGR α -F444V (c.1330 T>G, p.F444V) was obtained using the QuikChange Site-
9 Directed Mutagenesis kit (Stratagene, La Jolla, CA, United States) with pcDNA3-hGR α
10 (NM_000176.2) as a template.

12 **Transactivation assay**

13 HEK 293T cells were transfected with plasmids expressing wild-type (pcDNA3-hGR α) or mutated
14 GR (pcDNA3-hGR α - F444V; 40 ng/well of 96-well plates), a reporter Mouse Mammary Tumor
15 Virus-luciferase plasmid (MMTV-luciferase, 40 ng/well) and pMIR β -galactosidase (β -gal)
16 encoding the β -gal used to normalize luciferase activity (35 ng/well), using Fugene 6 (Promega)
17 following the supplier guidelines. Briefly, on the day of transfection, the medium was replaced by
18 a medium with 1% of FBS. After overnight incubation, the transfection medium was replaced by
19 a steroid-free charcoal-stripped medium. Twenty-four hours post-transfection, cells were treated
20 with vehicle or increasing dexamethasone concentrations (0.1–100 nM). As previously described,
21 luciferase and β -gal activities were measured from cell lysates³¹.

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1 **Western blot analysis**

2 As previously described³², total protein extracts were prepared from cells lysed at 4°C.
3 Immunoblots were incubated overnight at 4°C with an anti-GR antibody (Santa Cruz
4 Biotechnology Cat# sc-393232, [RRID:AB_2687823](#)) and the loading control (Sigma-Aldrich Cat#
5 A2066, [RRID:AB_476693](#)). Membranes were incubated at room temperature for 45 min with
6 secondary antibodies (Thermo Fisher Scientific Cat# 35518, [RRID:AB_614942](#) or 800 Cat# SA5-
7 35571, [RRID:AB_2556775](#)). Bands were visualized and quantified using Odyssey®Fc, Dual-
8 Mode Western Imaging (Li-Cor, Lincoln, NE, United States). Results are expressed as the median
9 [Q1; Q3] of 3 independent samples from 3 independent experiments.

10

11 **Immunofluorescence and 3D deconvolution microscopy**

12 Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized
13 for 30 min with a 0.5% solution of phosphate-buffered saline (PBS)–Triton X-100. Cells were then
14 washed 3 times with PBS and incubated for 1 h at room temperature in PBS/Tween 20 0.1% (v/v)
15 buffer containing 5% nonfat dry milk to reduce non-specific binding. Consecutively, cells were
16 incubated overnight at 4°C with a mouse anti-GR antibody (Santa Cruz Biotechnology Cat# sc-
17 393232, [RRID:AB_2687823](#)), followed by several washing with PBS/Tween 20 0.1% and
18 incubation with an Alexa Fluor 555 plus anti-mouse secondary antibody for 30 min at room
19 temperature (Thermo Fisher Scientific Cat# A32727, [RRID:AB_2633276](#)). After antibody
20 labeling, cells were post fixed for 10 min with 4% paraformaldehyde, then washed 3 times with
21 PBS, and counterstained with 0.5 µg/mL DAPI. Fluorescence microscopy was performed with an
22 automated upright BX61 microscope (Evident.olympus, Rungis, France) at 20x objective lens
23 (1.4NA) with the Image Pro Plus AMS software (Media Cybernetics Inc., Marlow, United

1 Kingdom) and a Mono Q Imaging Retiga 2000R Fast 1394 camera (Q Imaging Inc., Surrey, British
2 Columbia, Canada).

3

4 **Statistical analyses**

5 Data are expressed as mean \pm SEM when normal distribution was validated using the D'Agostino–
6 Pearson test. Data are described as median and interquartile range [Q1; Q3] if they do not follow
7 a normal distribution. All experiments were carried out at least twice independently of each other.
8 We used multiple Mann–Whitney tests with a Bonferroni correction (GraphPad Prism 10.0.1, San
9 Diego, CA, United States) for grouped analyses. *P* values <0.05 were considered statistically
10 significant. When independent experiments were pooled, the Bonferroni correction adjusted the *P*
11 value.

12

13 **Results**

14 **Case study**

15 A 68-year-old man was first seen at the Institute of Endocrinology at the Tel Aviv-Sourasky
16 Medical Center at the request of the Neurosurgery Department, to which he had been referred with
17 the diagnosis of Cushing's disease. His medical history included hypertension from the age of 21,
18 a sleeve gastrectomy at 59, a left adrenalectomy at 63, and osteoporosis at 65. Hypercortisolism
19 was first detected in 2013, during a routine screening to exclude Cushing's syndrome, which was
20 required before bariatric surgery. At that time, his BMI was 39.9 kg/m², with no clinical signs of
21 cortisol excess. Plasma potassium concentrations were within the normal range, and his blood
22 pressure was well controlled with amlodipine 10 mg daily. Urinary free cortisol (UFC) excretion
23 was 1415 nmol/24 h (normal up to 208 nmol/24 h), the morning serum cortisol after overnight

1 (O/N) administration of 1 mg dexamethasone was 264 nmol/L, and midnight salivary cortisol was
2 within the normal range. Baseline morning serum cortisol was 713 nmol/L, and basal plasma
3 ACTH was normal at 7.8 pmol/L (normal up to 10 pmol/L). The O/N 8 mg dexamethasone
4 suppression test was normal (cortisol 48 nmol/L), and a Dexamethasone-corticotropin releasing
5 hormone (Dex-CRH) test suggested NNH; therefore, the treating endocrinologist had agreed to
6 bariatric surgery, resulting in a weight loss of 33 kg. In 2017, he underwent excision of an enlarging
7 left adrenal mass, which on pathological examination was consistent with adenomatoid cortical
8 hyperplasia. No further hormonal investigations were performed at that time. After adrenalectomy,
9 plasma ACTH levels increased significantly, with persistently elevated serum and urinary cortisol
10 levels (Figure 1).

11 Two years before the current presentation, the patient received treatment with ketoconazole up to
12 1200 mg daily to reduce cortisol secretion. During the treatment period, there was a pronounced
13 increase in blood pressure, accompanied by leg edema. UFC dropped to 289 nmol/24 h, but
14 treatment was discontinued due to side effects. A possible pituitary lesion was identified on MRI;
15 therefore, the patient was referred for pituitary surgery, at which point we were consulted.

16 At the endocrine clinic, the patient reported long-standing irritability and restlessness, fatigue, and
17 muscular pain that increased with physical exercise. There was no history of easy bruising or
18 fragility fractures. On physical examination, the blood pressure was 131/74 mmHg, and BMI was
19 27.4 kg/m². There were no clinical signs suggestive of hypercortisolism. Complete blood count,
20 glucose and electrolytes, renal and liver function tests, and CPK levels were normal. Baseline
21 cortisol and ACTH levels were elevated, adrenal androgens were normal, and plasma renin activity
22 and aldosterone were suppressed (Table 1). Considering GGRS, we continued to test the reactivity
23 of the HPA axis to higher doses of dexamethasone³, an O/N 2 mg dexamethasone suppression test

1 was therefore performed. This test resulted in a decrease in ACTH and adrenal steroids; however,
2 cortisol did not fall below the threshold 50 nmol/L³³ (Table 1).

3 The absence of clinical signs of hypercortisolism, early-onset hypertension, adrenal hyperplasia,
4 and preserved diurnal cortisol secretion further supported the clinical diagnosis of GGRS.

5

6 **Genetic testing**

7 A heterozygous *NR3C1* variant (NM_000176.3) c.1330T>G p. Phe444Val was detected in the
8 proband (Figure 2A). This missense variant replaces the conserved amino acid phenylalanine with
9 valine at the base of the first zinc finger in the DNA binding domain (DBD, Figure 2B). Prediction
10 tools indicated a deleterious effect on the protein. This variant was not found in the general
11 population in the gnomAD database nor the Franklin database (comprising data from at least 10000
12 patient and control exomes and genomes). The variant has been classified as likely pathogenic by
13 the ACMG guidelines³⁴. Analysis of whole-exome sequencing data revealed no other pathogenic
14 or likely pathogenic variants. The patient did not carry any of the known polymorphisms of the
15 *NR3C1* gene (ER22/23EK, N363S, exon 9b-GR-9b) that have previously been associated with
16 modulation of glucocorticoid sensitivity³⁵. The proband has three daughters and one son. Daughter
17 H, aged 37, had class 3 obesity and underwent bariatric surgery at the age of 25. She also reports
18 hypertension, hirsutism, and mood swings. The other two daughters and son are in good health.
19 The proband's brother has also been diagnosed with obesity and hypertension (Figure 3).
20 Subsequent Sanger sequencing of the proband's three daughters confirmed that daughter H is a
21 carrier of the *NR3C1* variant, while her sisters Y and G are non-carriers. The son, T, refrained from
22 performing the test.

1
2 **GR_{F444V} displays a lack of transcriptional activity *in vitro***
3 To assess the transcriptional activity of GR_{F444V}, we designed expression vectors comprising either
4 GR_{F444V} or GR_{WT} that were transiently transfected into HEK 293T cells, as endogenous GR
5 expression is almost null. We first assessed GR_{F444V} protein expression by Western Blot, which
6 showed no difference between GR_{WT} and GR_{F444V} expression after transfection (Figures 4A and
7 B, GR_{WT} 1.03 [0.9;1.1] vs GR_{F444V} 1.2 [0.9; 1.7], $P=0.42$). In response to increasing
8 dexamethasone concentrations, GR_{WT} transactivated the luciferase reporter gene, with maximum
9 transactivation at 10 nM dexamethasone (Figure 4C, relative transactivation at DEX 10 nM 91.5
10 [80.5;101.2]). In contrast, the GR_{F444V} showed no transactivation capacity, even in response to 100
11 nM DEX (Figure 4C, relative transactivation at DEX 100 nM 1.0 [1.0;1.0]). GR_{F444V} exerted a
12 dominant negative effect when transfected in equal amounts with GR_{WT} (Figure 4D, GR_{WT} 96.8
13 [88.8;112.6] vs GR_{WT}/GR_{F444V} 48.9 [38.9;59.4], $P < 0.001$). Considering the nuclear localization
14 signal within the DNA-binding domain, we assessed nuclear translocation in response to
15 dexamethasone using immunodetection of GR_{WT} and GR_{F444V} in HEK 293T transfected cells. Both
16 wild-type and mutated GR were mainly localized in the cytoplasm in the absence of hormones.
17 We observed a conserved nuclear translocation of the GR_{F444V} variant in response to 100 nM
18 dexamethasone (Figure 5), indicating that the defect of transcriptional activity is due to impaired
19 DNA binding rather than reduced nuclear translocation.

20

21 **Three-dimensional model of the GR DNA-binding domain (GR-DBD)**

22 Phe444 is located within the DBD, specifically at the base of the first zinc finger. This
23 Phenylalanine residue is conserved across all species and in all nuclear receptors. The 3D structure

1 of the DNA-bound DBD indicates that this phenylalanine is involved in multiple Van der Waals
2 interactions stabilizing the structure (Figure 6A and B, PDB 1R4O). As shown in Figure 6C,
3 substituting Phe444 with a shorter valine drastically reduces the number of contacts, likely
4 resulting in a poorly or non-structured DBD. The significance of Phe444 was highlighted in a site-
5 directed mutagenesis study by Schena et al., which showed that replacing Phe444 with serine
6 impaired DNA binding³⁶.

7

8 **Discussion**

9 We identified a novel missense heterozygous variant of the *NR3C1* gene associated with familial
10 primary glucocorticoid resistance syndrome. This syndrome is characterized by elevated cortisol
11 levels with normal or elevated ACTH without the classic signs of Cushing syndrome³. It is a rare
12 disease with extremely variable phenotypes, ranging from asymptomatic to resistant hypertension
13 and hypokalemia. The differential diagnosis between GGRS and ACTH-dependent neoplastic
14 hypercortisolism is difficult to establish. Although patients with GGRS lack the more specific
15 signs of Cushing syndrome such as skin atrophy, striae, muscle wasting and dorsocervical fat, they
16 may present with several common manifestations of cortisol excess including early-onset
17 hypertension, hypokalemia and obesity, as well as ACTH-driven effects leading to
18 hyperandrogenism in women and adrenal hyperplasia. GGRS has been included among the
19 etiologies that can cause NNH with Cushing phenotype, along with psychiatric disorders, poorly
20 controlled diabetes mellitus, alcoholism, and advanced chronic kidney disease³⁷. The diagnostic
21 challenge is further complicated by the increasing recognition of patients with milder forms of
22 Cushing syndrome without classical stigmata³⁸. Notably, diabetes mellitus and osteoporosis have

1 rarely been diagnosed in patients with GGRS. Although the latter has not been systematically
2 investigated, no cases of fragility fracture have been reported in these patients. Finally, mild forms
3 of GGRS are probably more common than previously thought, reaching a prevalence of 5% in
4 patients incidentally found to have bilateral adrenal adenomas/hyperplasia that were screened for
5 the presence of *NR3C1* mutations⁸. Interestingly, the prevalence of hypertension was higher in
6 non-mutated (89.5%) than in mutated patients (40%).

7 The Dex/CRH test has high diagnostic accuracy in differentiating Cushing syndrome from NNH³⁹,
8 but its performance in GGRS is unknown, as it was only performed in our patient. Another valuable
9 test used for the differential diagnosis of NNH and Cushing syndrome is the cortisol and ACTH
10 responses to desmopressin administration³⁷. However, the use of this test has not been reported in
11 GGRS patients. A preserved circadian cortisol rhythm emerges as the most reliable laboratory
12 diagnostic tool for differentiating between GGRS and Cushing syndrome. Remarkably, this is also
13 true in cases of extremely elevated serum cortisol levels as illustrated by the patient reported by
14 Lamberts et al.³⁹, who presented with a morning cortisol of 4102 nmol/l and a cortisol level of
15 1840 nmol/l at 10 pm. Alwani et al., reported that cortisol midnight/morning ratios > 0.67 are
16 suggestive of Cushing disease with a positive predictive value of 100% and a negative predictive
17 value of 73%, when compared to patients with NNH⁴⁰. In all 15 cases of GGRS reported, cortisol
18 rhythmicity was preserved. In nine patients, it was possible to calculate the ratio of evening to
19 morning serum cortisol. The median value was 0.32 (IQR 0.14-0.39), and the highest value was
20 0.44, all well below the cut-off of 0.65. Although some cortisol values were obtained in the late
21 evening, the ratios calculated from these values can be expected to be higher than if sampling had
22 taken place at midnight, confirming the validity of the results. In four cases, salivary cortisol levels
23 at midnight were within the normal range.

1 In the family, reported herein, the two patients carrying the mutation and the untested proband's
2 brother presented with hypertension and obesity. Hypertension is a frequent and characteristic
3 symptom of primary GGRS, while obesity is generally not considered a component of this
4 syndrome³. According to our review, high blood pressure was present in 43.1% of patients, while
5 45.7% were overweight or obese. Similar frequencies were reported by Vitellius et al⁴¹. In this
6 patient, suppressed plasma renin activity and aldosterone levels are consistent with the activation
7 of MR by cortisol and other steroid precursors. Additionally, considering the patient's presentation
8 of leg edema and exacerbated hypertension associated with ketoconazole therapy, we hypothesized
9 that these symptoms could be attributed to the inhibition of steroidogenic enzymes by
10 ketoconazole. This inhibition likely led to increased steroids such as corticosterone and 11-
11 deoxycorticosterone, which can activate the mineralocorticoid receptor (MR) despite the observed
12 decrease in cortisol secretion. Notwithstanding, the patient did not have hypokalemia, similarly to
13 half of GGRS patients with hypertension.

14 In contrast, obesity is usually associated with glucocorticoid hypersensitivity⁴². Given that the
15 proband's two healthy daughters do not exhibit obesity, it can be inferred that this phenotype is
16 related to the mutation, although causality cannot be definitively established. In human adipose
17 tissue, glucocorticoids induce adipocyte differentiation, increasing adiposity and insulin resistance
18 through GR binding⁴³. Nevertheless, it was demonstrated that mice with adipocyte-specific GR
19 deficiency developed corticosterone-induced obesity without metabolic complications⁴⁴. It has
20 also been shown that MR mRNA expression is increased in fat tissue of obese patients and mice,
21 and a positive correlation was observed between BMI and MR mRNA levels, whereas no such
22 correlation was found with GR mRNA levels⁴⁵. These findings suggest that activation of the MR
23 by glucocorticoids might play a role in adipogenesis and processes linked to adipose tissue

1 dysfunction. These mechanisms could become predominant in patients with loss-of-function
2 variants of the *NR3C1* gene. On the other hand, the obesity observed in mutated patients may be
3 explained by tissue-specific regulation of GR function. Studies in tissue-specific GR knockout
4 mice have shown that GR expression in adipose tissue is essential for diet-induced obesity⁴⁶.
5 Therefore, it could be hypothesized that elevated plasma cortisol levels might stimulate lipogenesis
6 without causing other signs of Cushing's syndrome, especially if adipose tissue retains some
7 sensitivity to glucocorticoids in these patients. Our team previously reported a case involving a
8 man who showed reversible glucocorticoid resistance at the pituitary level, consistent with the
9 tissue-specific nature of glucocorticoid sensitivity³². Consequently, in this case, endogenous
10 glucocorticoids might also effectively overcome resistance in adipocytes³². In contrast to the
11 complete lack of transactivation observed in our *in vitro* experiments, even at high dexamethasone
12 concentrations, inhibition of the HPA axis activity in our patient was dose-dependent and achieved
13 with increasing doses of dexamethasone, emphasizing the limited correlation between *in vitro*
14 functional characterization of the GR and clinical manifestations observed in patients. Indeed,
15 treatment with high doses of dexamethasone consistently improved CRH and ACTH-dependent
16 clinical manifestations. Ma *et al.* reported a significant decrease in adrenals (55.6% and 32.4%)
17 and pituitary volumes (18.9%) following two years of dexamethasone treatment⁴⁷. Similarly, a
18 female patient diagnosed with delayed puberty and ambiguous genitalia attributed to
19 glucocorticoid resistance syndrome responded well to treatment with dexamethasone 6 mg/day⁴⁸.
20 In such cases, high-dose dexamethasone appears capable of partially activating the mutated GR.
21 This activation leads to decreased CRH, ACTH and adrenal androgens secretion without causing
22 mineralocorticoid effects. In contrast, excision of the adrenal mass in our patient (Figure 1)
23 resulted in a significant increase in ACTH secretion, which restored serum and UFC levels to pre-

1 adrenalectomy levels. Intriguingly, as reported here for the first time, ACTH levels did not stabilize
2 at this new steady state but continued to increase progressively. Whether this is accompanied by
3 an increase in pituitary volume, and what the consequences might be, remains to be determined.
4 Interestingly, in one case, a patient diagnosed with GGRS, developed a 9 mm corticotroph
5 adenoma several months after bilateral adrenalectomy⁴⁹.

6 Here we showed that the variant GR_{F444V} cannot transactivate the MMTV promoter in response to
7 dexamethasone and exerts a dominant negative effect on the WT receptor. Nuclear translocation
8 was preserved at 1 h, suggesting that the GR_{F444V} is able to bind dexamethasone and translocate to
9 the nucleus. As the amino acid mutated is located between the two zing fingers in an area
10 associated with DNA binding, we hypothesized that the DNA binding of the GR_{F444V} is impaired.
11 This is consistent with a publication by Schena et al.³⁶, in which the authors mutated different
12 amino acids, including the corresponding phenylalanine, from DBD and assessed DNA binding
13 by gel retardation assay³⁶. Mutation of Phenylalanine at position 444 resulted in an absence of
14 DNA binding and transactivation activity, supporting our findings³⁶. Further, phenylalanine is a
15 well-conserved aromatic amino acid between species and nuclear receptors. As shown in Figure
16 5, valine fails to establish numerous van der Waals contacts that could stabilize the DBD structure
17 and enable DNA binding. DBD is also involved in dimer formation through the D-loop. It can
18 therefore be assumed that the variant may modify GR dimerization⁵⁰. Consistent with this
19 hypothesis, a dominant negative effect, mediated by GR occupancy by an inactive receptor or by
20 the formation of inactive dimers, has been described in several glucocorticoid-resistant variants
21 located within LBD, but not in DBD^{51–53}. Interestingly, we observed a dominant negative effect
22 of the variant on the WT receptor (Figure 3D), suggesting a conserved dimerization capacity,
23 which could be explained by the localization of the mutation away from the D-loop. Taken

1 together, these data suggest that the Phe444 is critical for DNA binding but not for dimerization.
2 Numerous patients exhibit phenotypes associated with haploinsufficiency, in which glucocorticoid
3 signaling is partially restored by expression of the WT receptor. This was nicely demonstrated by
4 Bouligand et al., in patients expressing the GR_{R469X}, showing partial rescue of GR activity in
5 response to dexamethasone in fibroblasts from patients expressing both the mutated and the WT
6 allele³¹. Unfortunately, we were unable to assess protein expression and activity in patient
7 fibroblasts. Nevertheless, considering the dominant negative effect, we could hypothesize that
8 glucocorticoid signaling is only partially restored by expression of the WT allele.

9 To conclude, we have described a novel heterozygous variant of the *NR3C1* gene causing familial
10 primary glucocorticoid resistance. This case underscores the diagnostic challenges and prolonged
11 delays in diagnosis, resulting in unnecessary surgeries for patients and potentially affected family
12 members. Additionally, this study highlights several clinical and laboratory features of GGRS that
13 should alert physicians to consider this rare diagnosis in patients presenting with NNH.

14

15 **Acknowledgments**

16 We wish to thank Dr. Dima Namouz for her dedicated care of this patient.

17

18 **Data availability**

19 Some or all datasets generated during and/or analyzed during the current study are not publicly
20 available but are available from the corresponding author on reasonable request.

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1 **Figure Legends**

2 **Figure 1: Plasma ACTH, serum cortisol and 24-hour urinary free cortisol (UFC) post-**
3 **adrenalectomy.**

4 **Figure 2: Localization of the missense variant within the DBD**

5 A) Schematic representation of the *NR3C1* gene with the location of the missense variant
6 within the third exon.

7 B) Schematic representation of the DNA-Binding Domain with the location of the amino acid
8 substitution in red.

9 **Figure 3: Proband family tree**

10 Representation of the patient's family tree, with associated clinical features (dark grey:
11 hypertension, black: obesity, grey: mood disorders, light grey: hirsutism) and the variant
12 represented by a black dot.

13 **Figure 4: Effect of GR_{F444V} variant on transactivation activity**

14 A) Expression of GR protein (90 kDa) in transiently transfected HEK 293T cells. Lysate and
15 protein extract from transfected cells were analyzed using SDS-PAGE. GR proteins were
16 detected by Western Blot analyses using a mouse antibody targeting the N-terminal domain
17 of hGR α . β -actin was used as a loading control. A representative image of 3 independent
18 experiments is shown.

19 B) Quantification of protein level. Specific bands were quantified by Odyssey FC, Dual
20 Mode Western Imaging. Results are expressed as median [Q1; Q2] and normalized to β -
21 actin loading, with control condition values arbitrarily set at 1. Data are expressed from

1 individual samples from 3 independent experiments. Statistical analysis was performed
2 using Mann Whitney t-test with Bonferroni correction, NS, $P = 0.42$.

3 C) HEK 293T cells were transiently transfected with plasmids encoding GR_{WT} or GR_{F444V}
4 (40 ng), a luciferase reporter plasmid pMMTV-Luc (40 ng) and pMIR β gal (35 ng). Cells
5 were treated 24 h with increasing dexamethasone concentrations (0.1 to 100 nM) in a
6 serum-free medium. Transactivation activity is expressed relatively to the maximum
7 transactivation activity obtained for GR_{WT} with an agonist concentration of 100 nM, set
8 arbitrarily at 100%. Data are expressed as non-linear regression from 2 independent
9 experiments performed in 8 replicates.

10 D) HEK 293T cells were transiently transfected with 30 ng of GR_{WT} and increasing amounts
11 of plasmid encoding GR_{F444V} (0 to 30 ng) combined with luciferase reporter plasmid
12 pMMTV-Luc (40 ng) and pMIR β gal (35 ng). Cells were treated for 24 h with 1 nM
13 dexamethasone in a serum-free medium. Transactivation activity was expressed relatively
14 to the maximum transactivation activity obtained for 30 ng GR_{WT} at 1 nM dexamethasone,
15 arbitrarily set at 100%. Data are expressed as median [Q1; Q3] from 8 replicates of 2
16 independent experiments. Statistical significance was calculated with the Multiple Mann-
17 Whitney test with Bonferroni correction, **** $P < 0.0001$.

18
19 **Figure 5: Nuclear translocation of the GR_{WT} and GR_{F444V} in response to dexamethasone**

20 COS-7 cells were transiently transfected with plasmids encoding GR_{WT} or GR_{F444V}
21 (400 ng). Transfected cells were incubated with 0 or 100 nM dexamethasone for 24 h after
22 transfection. Cells were fixed and processed for immunochemistry as described in the

1 Materials and Methods section. Representative images of GR subcellular localization (red).
2 DAPI staining delineates nuclei (blue). The nuclear localization of GR is shown in the
3 merged panel (pink).

4
5 **Figure 6: Three-dimensional modeling of the GR_{F444V} DBD**

6 A) Picture showing the overall organization of the dimerized DNA-bound GR-DBD (PDB
7 1R4O). The α -helices and beta-sheets are depicted as ribbons and arrows, respectively.
8 Selected residues are shown with their carbon, oxygen, and nitrogen atoms colored in
9 white, red, and blue, respectively.

10 B) The focus is on the hydrogen bonds network involving Phe444 with amino acids located
11 either between the zinc fingers (Ala448, Tyr455), in the second zinc finger (Pro474), or
12 after the second zinc finger (Arg477, Tyr478, Cys481, and Ala546).

13 C) Focus showing the loss of hydrogen bonds after substitution of Phe444 to Val444,
14 except for Arg477.

1

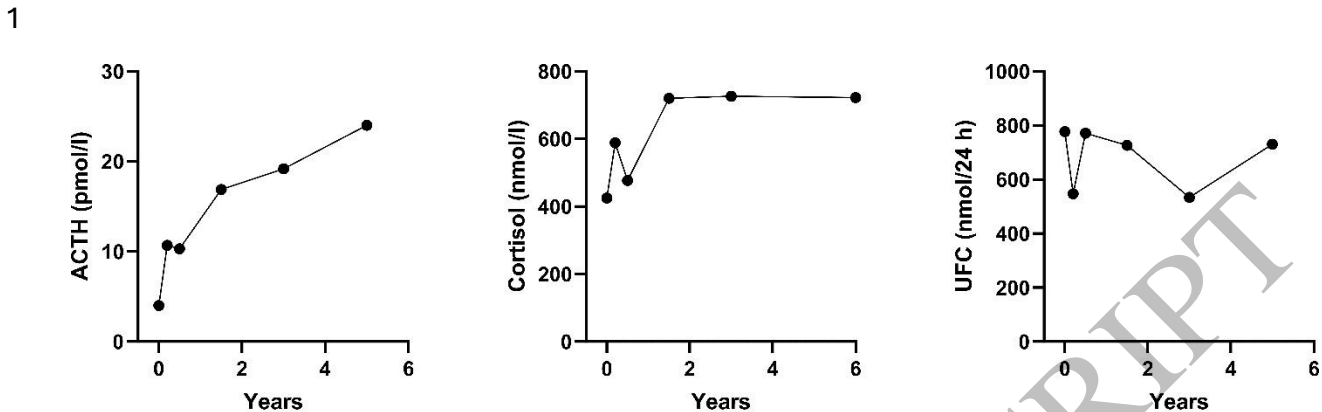
2 **Table 1. Adrenal steroids and ACTH response to overnight dexamethasone 2 mg suppression**

3 **test**

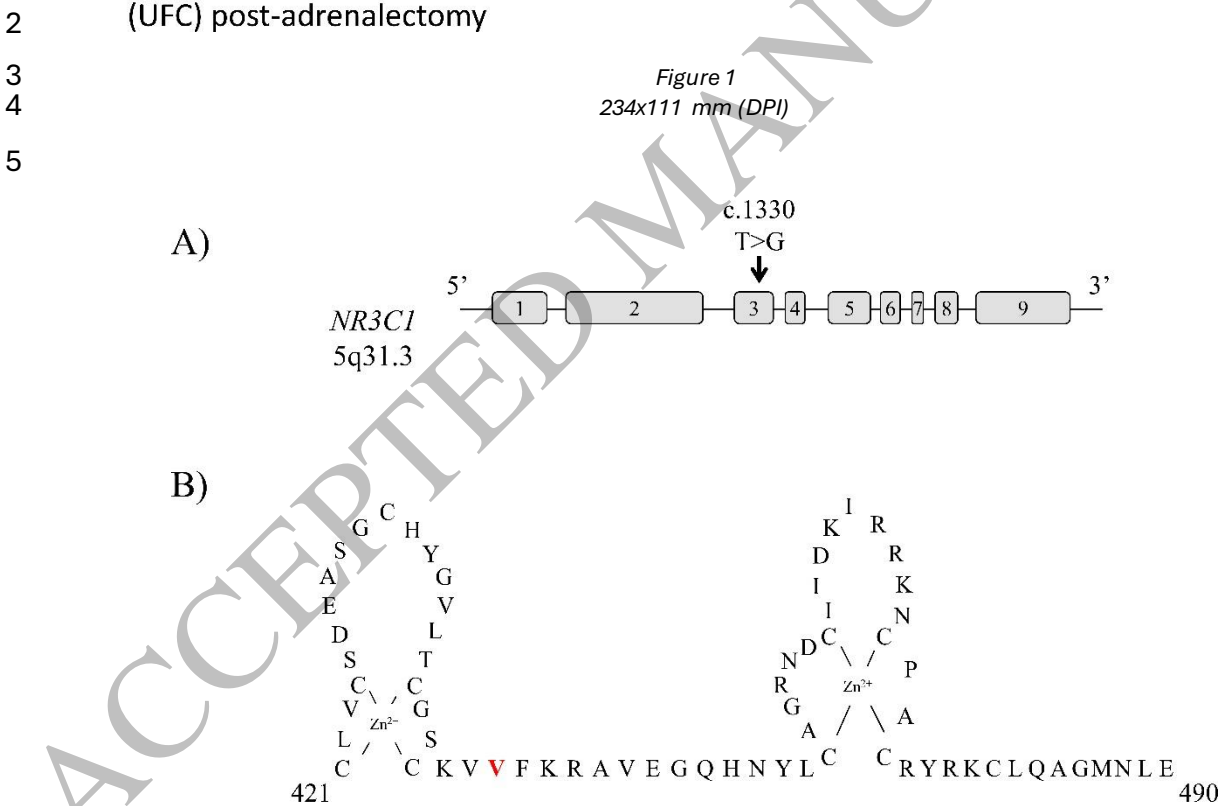
	Baseline	After 2 mg dexamethasone
ACTH (pmol/l) (1.3 – 16.7)*	16.4	2.1
Cortisol (nmol/l) (140 – 690)	772	51.6
Androstenedione (nmol/l) (1.4 – 7.63)	7.2	1.7
DHEAS (µmol/l) (1.13 – 7.83)	2.9	2.4
Aldosterone (nmol/l) (0.2 – 0.8)	<0.02	<0.02
Plasma renin activity (ng/ml/hr) (0.7 – 3.3)	0.49	0.52

4 ACTH- Adrenocorticotropin hormone; DHEAS- dehydroepiandrosterone sulphate

5 *Numbers in parenthesis indicate the normal range



2 Figure 1. Plasma ACTH, serum cortisol and 24-hour urinary free cortisol
3 (UFC) post-adrenalectomy



6 Figure 2. Localization of the missense variant within the DBD

7 Figure 2
8 138x94 mm (DPI)

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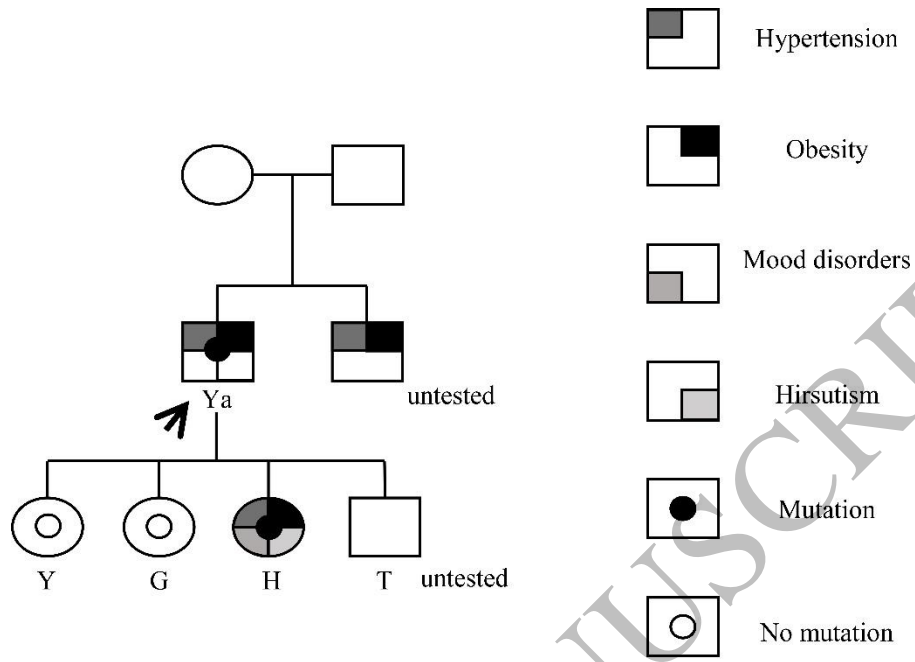


Figure 3. Proband family tree

Figure 3
119x103 mm (DPI)

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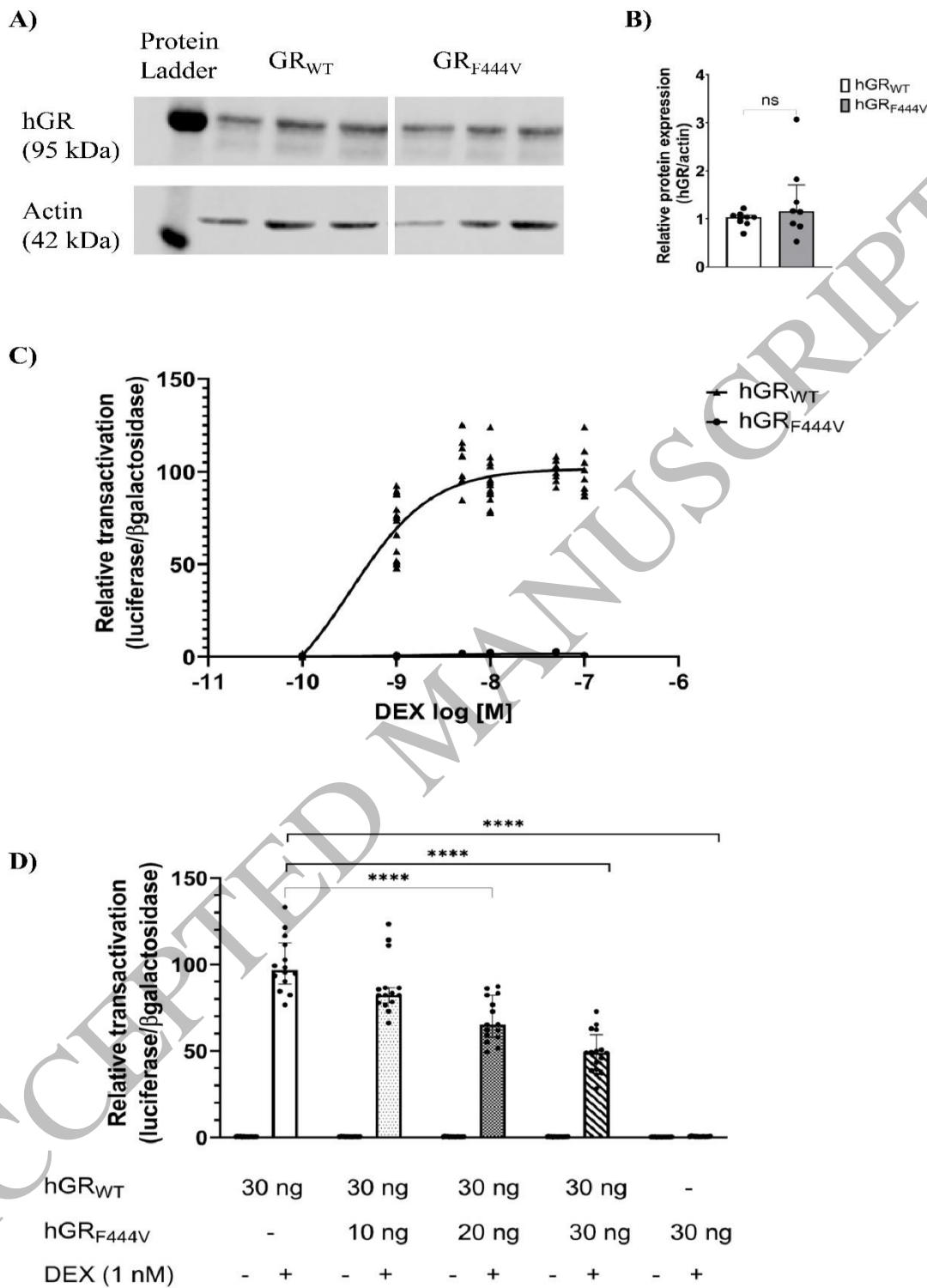
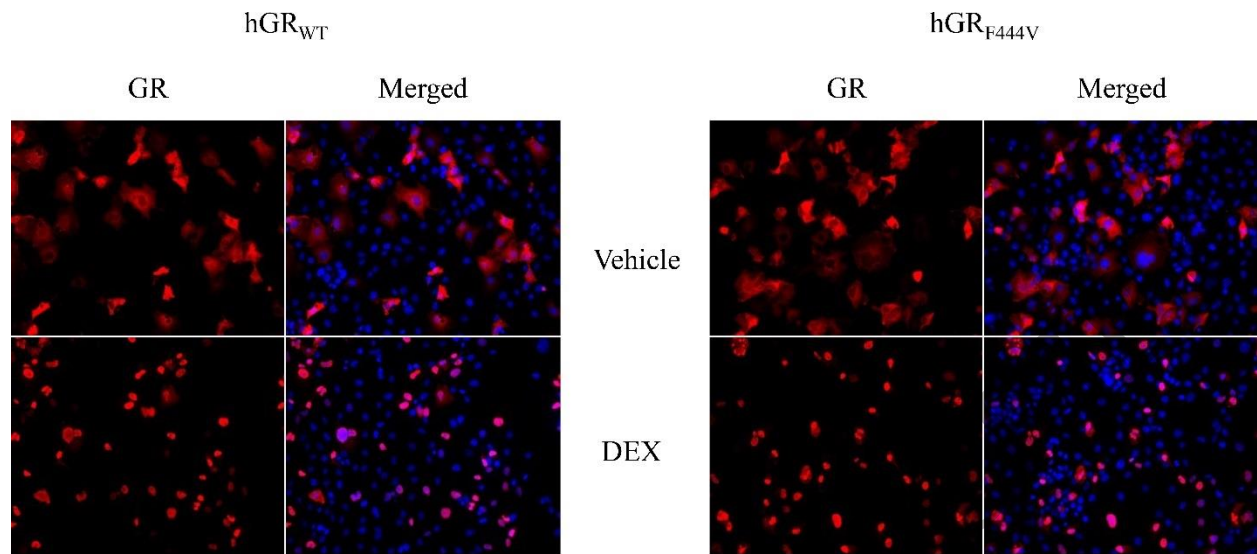


Figure 4. Effect of the GR_{F444V} variant on transactivation activity

Figure 4
142x243 mm (DPI)

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1 Figure 5. Nuclear translocation of the GR_{WT} and GR_{F444V} in response to dexamethasone

2 *Figure 5*
 3 *181x93 mm (DPI)*

4

ACCEPTED MANUSCRIPT

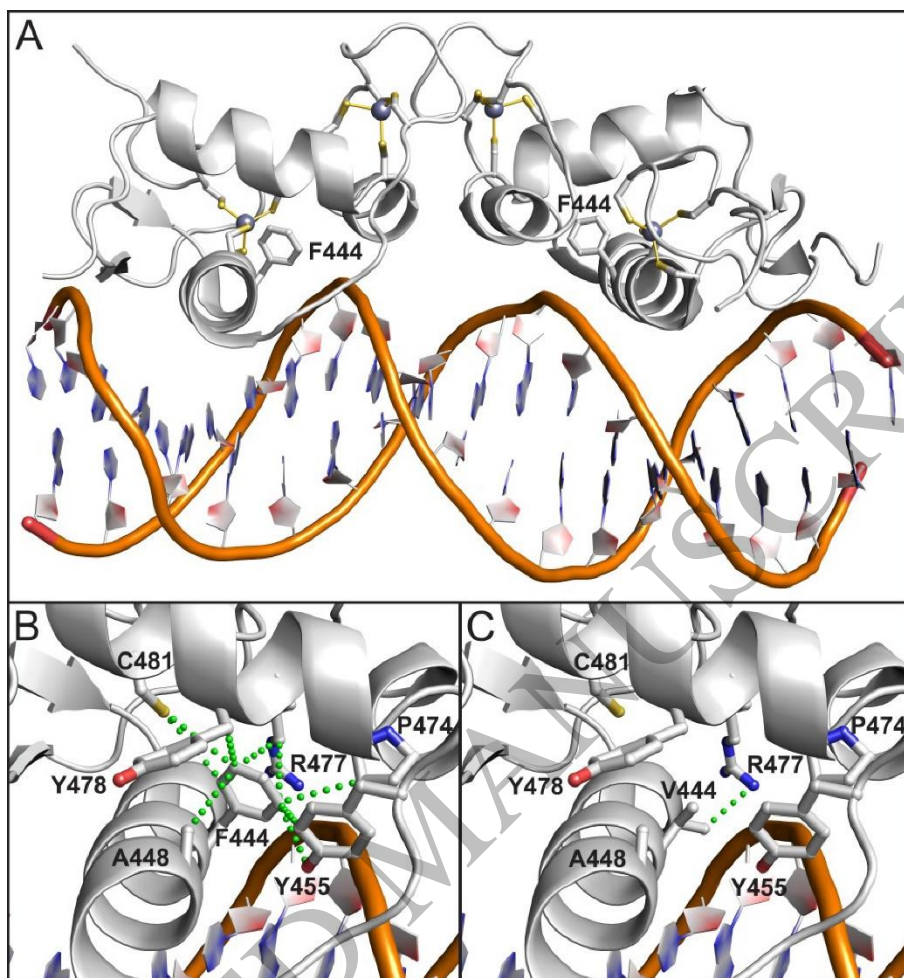


Figure 6. Three-dimensional modeling of the GR_{F444V} DBD

Figure 6
120x139 mm (DPI)

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