# Significant prevalence of *NR3C1* mutations in incidentally discovered bilateral adrenal hyperplasia: results of the French MUTA-GR Study

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European Journal of Endocrinology

## Abstract

*Background*: Recently discovered mutations of *NR3C1* gene, encoding for the GR, in patients with glucocorticoid resistance and bilateral adrenal incidentalomas prompted us to investigate whether GR mutations might be associated with adrenal hyperplasia.
*Objective*: The multicenter French Clinical Research Program (Muta-GR) was set up to determine the prevalence of GR mutations and polymorphisms in patients harboring bilateral adrenal incidentalomas associated with hypertension and/or biological hypercortisolism without clinical Cushing's signs.
*Results*: One hundred patients were included in whom *NR3C1* sequencing revealed five original heterozygous GR mutations that impaired GR signaling *in vitro*. Mutated patients presented with mild glucocorticoid resistance defined as elevated urinary free cortisol ($1.7 \pm 0.7$ vs $0.9 \pm 0.8$ upper limit of normal range, $P = 0.006$), incomplete 1 mg dexamethasone suppression test without suppressed 8-AM adrenocorticotrophin levels ($30.9 \pm 31.2$ vs $16.2 \pm 17.5$ pg/mL) compared to the non-mutated patients. Potassium and aldosterone levels were lower in mutated patients ($3.6 \pm 0.2$ vs $4.1 \pm 0.5$ mmol/L, $P = 0.01$, and $17.3 \pm 9.9$ vs $98.6 \pm 115.4$ pg/mL, $P = 0.0011$, respectively) without elevated renin levels, consistent with pseudohypermineralocorticism. *Ex vivo* characterization of mutated patients' fibroblasts demonstrated GR haploinsufficiency as revealed by below-normal glucocorticoid induction of *FKBP5* gene expression. There was no association between GR polymorphisms and adrenal hyperplasia in this cohort, except an over-representation of *BclI* polymorphism.
*Conclusion*: The 5% prevalence of heterozygous *NR3C1* mutations discovered in our series is higher than initially thought and encourages GR mutation screening in patients with adrenal incidentalomas to unambiguously differentiate from Cushing's states and to optimize personalized follow-up.

*European Journal of Endocrinology*
(2018) **178**, 411–423

## Introduction

Glucocorticoids are fundamental hormones that regulate various biological functions involved in development, metabolism, inflammatory processes and stress. Their actions are mediated by the glucocorticoid receptor (GR), an intracellular receptor protein that functions as ligand-activated transcription factor (1). Human GR

www.eje-online.org
https://doi.org/10.1530/EJE-17-1071
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(hGR), encoded by the *NR3C1* gene (MIM#138040), located in the chromosome 5, comprises 10 exons. Alternative splicing of the hGR gene in exon 9 generates two homologous receptor isoforms: α and β (2). Exon 2 encodes for the N-terminal domain and exons 3 and 4 encode for the DNA-binding domain (DBD). Exons 5–9 encode for the ligand-binding domain (LBD). *NR3C1* loss-of-function mutations have been associated with a glucocorticoid resistance syndrome (MIM#615962). Moreover, among many *NR3C1* polymorphisms described to date, some were associated with increased (N363S, *BclI*) or decreased (ER22/23EK) glucocorticoid sensitivity, adrenal incidentalomas (ER22/23EK, *BclI*, 9β), obesity and metabolic syndrome (N363S, *BclI*, 9β) (3, 4).

Twenty-four loss-of-function germinal mutations in *NR3C1* have been reported and shown to impair intracellular GR signaling (5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15). Fifty percent of mutated probands presented with hypertension, frequently associated with hypokalemia, while the majority of mutated women presented with hirsutism. GR mutation carriers had elevated or normal adrenocorticotrophin (ACTH) levels associated with high urinary free cortisol (UFC) and lacked negative glucocorticoid feedback loop on the hypothalamic–pituitary adrenal (HPA) axis.

We recently discovered the first nonsense heterozygous mutation of human *NR3C1* (MIM #615962), R469X, in a context of bilateral adrenal hyperplasia incidentally discovered (16). The proband displayed no Cushing's syndrome signs but had high blood pressure with hypokalemia. Hormonal evaluations revealed a biological hypercortisolism with normal ACTH level. Functional characterization demonstrated GR haploinsufficiency. Moreover, in mouse models, Michailidou and coworkers (17) had shown that heterozygous $GR^{+/-}$ exhibited bilateral adrenal hyperplasia but normal plasma ACTH levels. Taken together, these data led us to hypothesize that impairment in GR signaling could be related to adrenocortical hyperproliferation and/or hyperplasia.

Bilateral adrenal incidentalomas represent 15–20% of adrenal incidentalomas (18, 19, 20). This prevalence drastically increases with aging. The best known causes of bilateral adrenal masses are related to macronodular adrenal hyperplasia, potentially regulated by aberrant G protein-coupled receptors (21), familial pheochromocytoma, primary hyperaldosteronism (22), *ARMC5* gene mutation (23, 24), 21-hydroxylase deficiency, multiple endocrine neoplasia type 1 (MEN-1), infectious diseases, non-functioning tumor or metastatic diseases (18). However, a direct relationship between adrenal incidentalomas and altered GR signaling has never been investigated thus far nor evoked in recent published guidelines (25, 26).

In the present study, we hypothesized that GR mutations might represent a novel genetic cause of adrenocortical proliferation in the context of autonomous glucocorticoid secretion. A French National, Hospital Clinical Research Program (PHRC), Muta-GR, was set up to determine the prevalence of *NR3C1* mutations in a cohort of patients presenting with bilateral adrenal incidentalomas combined with high blood pressure (HBP) and/or biological hypercortisolism without Cushingoid features. The second aim was at evaluating common GR polymorphisms frequency in our series associated with adrenal hyperplasia compared to the general population. We discovered five novel heterozygous *NR3C1* mutations, among which three have been already functionally characterized (16, 27). All reported mutations impaired GR signaling either by glucocorticoid or DNA-binding defects leading to a clear-cut GR haploinsufficiency. The relatively high prevalence of *NR3C1* mutations in our cohort emphasizes the importance of GR genetic screening in selected patients enabling their appropriate management and to optimize their follow-up strategies.

## Patients and methods

### Study protocol

The study referred to as Muta-GR (ClinicalTrials.gov Identifier: NCT02810496) is a multicenter and transversal PHRC study. The primary objective of this study was to evaluate the prevalence of *NR3C1* mutations in patients with bilateral adrenal incidentalomas. To refine the rate of GR genetic alterations, patients with hypertension and/or biological hypercortisolism without Cushing's syndrome signs were selected. Inclusion criteria were the presence of incidentally discovered bilateral adrenal hyperplasia in patients with no overt signs of Cushing's syndrome. Adrenal imaging was performed in patients for unrelated reasons. Adrenal hyperplasia was radiologically characterized and defined using morphological and volumetric criteria. To date, objective characteristics of adrenal hyperplasia are only poorly specified. In the present study, adrenal hyperplasia was defined according to three main criteria: adrenal limb (medial and lateral) >5 mm (28), adrenal volume above reference values in the general population (as referred in Carsin *et al.* (29)) or the presence of adrenal nodule >10 mm. The presence of hypertension was defined as a systolic blood pressure (SBP) >140 mm Hg, diastolic blood pressure (DBP) ≥90 mmHg





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and/or patients under any current antihypertensive therapy. Hypercortisolism was assessed based on either high UFC (above reference values or upper limit of normal range (ULN)), midnight serum cortisol >50 nmol/L (30) or serum 8-AM cortisol >50 nmol/L after 1 mg dexamethasone (DXM) suppression test (1 mg DST). Nine patients had UFC above ULN, 23 had cortisol >50 nmol/L after 1 mg DST while 58 had midnight serum cortisol >50 nmol/L. Cushing's syndrome signs were evaluated by clinician endocrinologists (thin skin, hirsutism, easy bruising, faciotruncal adiposity and amyotrophy). We excluded patients with clinical Cushing's syndrome, current cancer, age under 18 years or without written patients' consent.

The study protocol conformed to the ethics guidelines in accordance with the Declaration of Helsinki and was approved by the Comité de Protection des Personnes, CPP Est III on Aug 12, 2011.

## Patients

One hundred twenty-seven patients were enrolled between Aug 2012 and Oct 2016 in twenty endocrinology departments of French University Hospitals (see the list of investigators).

All investigators filled out forms listing sex, birth date, weight (kg), height (cm), SBP, DBP (mmHg) and cardiac disease (existence of heart failure or coronaropathy), dyslipidemia, current antihypertensive, anti-diabetic or anti-cholesterol medications. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters.

Biochemical and hormonal analyses were collected from each center: kalemia, creatininemia, creatiniuria, fasting glycaemia (8-AM), glycated hemoglobin (HbA1c), total cholesterol (g/L), HDL cholesterol (g/L), LDL cholesterol (g/L), UFC, 8-AM and midnight plasma cortisol, cortisol after 1 mg DST, 8-AM ACTH level, plasma aldosterone and renin concentrations in supine position.

## Imaging study

Multidetector row computed tomography (MDCT) examinations were performed in each different institution (see the list of investigators). Protocols for the acquisition of images covering the abdomen were variable in terms of intravenous administration of iodinated contrast agent and also in terms of scanning parameters, but they always included thin slices covering the abdomen. Anonymized MDCT data were transferred to a dedicated workstation equipped with Myrian 64 Expert VL1.15.0 software (Intrasense, Montpellier, France). The maximum width of the gland (defined as the maximum width perpendicular to the long axis of the body gland, at the junction of adrenal limbs and the body) and width of the adrenal limbs (defined as the maximum thickness of the medial and lateral limbs of the gland perpendicular to the long axis of the limb) were measured as previously described by Vincent *et al.* (31). The presence of adrenal nodules >10 mm, their precise size, attenuation when recordable and degree of enhancement were noted. Nodules suspicious for non-adenomas were excluded from the study, on the basis of the consensual criteria for the diagnosis of adenoma. Adrenal gland volume was calculated according to Carsin-Vu (29), on the same workstation. The adrenal contour used for volume calculation was traced semi-automatically for all patients by one investigator, after an intense training session with an expert radiologist. The software then automatically calculated adrenal volume by summing the area on each slice. Care was taken to exclude adjacent fat. Total adrenal volume (TAV) was determined as the sum of the right and left adrenal volume measurements and expressed in $cm^3$.

## DNA analysis

Each blood sample was sent to University Hospital of Kremlin Bicêtre. Genomic DNA was extracted from white blood cells. The entire coding regions of the hGRα and the hGRβ were amplified and sequenced by Sanger method with primers previously described (16). We also examined some GR SNPs: rs6189, rs6190, rs10482622, rs6195, rs41423247, rs6188 and rs6196.

## Fibroblast culture

After skin biopsy, primary cultures of patients' fibroblasts were grown at 37°C with 5% $CO_2$ in DMEM High Glucose (4.5 g/L) (Life Technology), 2 mM glutamine, 2 mM Hepes (GIBCO), 1 mM sodium pyruvate (GIBCO), 100 U/mL penicillin, 100 µg/mL streptomycin (GE Healthcare) and 15% fetal bovine serum (Biowest, Nuaillé, France).

## RT-real-time qPCR

Patients' fibroblasts were washed once with ice-cold PBS. Total RNA was extracted with the TRI-reagent (Molecular Research Center, Inc., Euromedex, Mundolsheim, France), according to the manufacturer's recommendations. Total





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RNA extraction, reverse transcription and real-time qPCR were performed essentially as previously described (32). cDNA samples were amplified by real-time qPCR by using the QuantStudio6 Flex System (Life Technologies). All samples were quantified in duplicate. Relative expression in a given sample was normalized to the internal reference rRNA 18S values, where the control condition values are arbitrarily set at 100%. Results are expressed as means ± S.E.M.

### Primer design for RT-qPCR assays

Primer pairs encompassing the targeted regions were designed by using NCBI's software Primer BLAST. Primers were provided from Eurogentec (www.eurogentec.com), purified by the selective precipitation optimized process (SePOP), desalted and delivered at 100 µM concentration in water. Primer sequences were as follows: h18S Forward: GTG CAT GGC CGT TCT TAG TTG, Reverse: TGA ATG CCA CAT CTC TGC AGT; hFKBP5 Forward: CCG GAG AAC CAA ACG GAA A, Reverse: TGA ATG CCA CAT CTC TGC AGT.

### Statistical analysis

Results are expressed as means ± S.D. in tables and means ± S.E.M. in figures. Statistical analyses were performed with Prism 5 software (GraphPad Software). Comparison of continuous variables among different groups was performed using Mann–Whitney *U* tests or Fisher *t* tests for categorical variables. *P* values of less than 0.05 were considered significant.

## Results

### Clinical, radiological and biochemical characteristics of eligible patients

One hundred and twenty-seven patients were initially enrolled. Eighteen patients were excluded after analysis of the MDCT by the radiologist because the abnormalities in terms of size and/or volume of the gland or of the presence of a macronodule were not bilateral. Nine patients were also excluded since they had neither hypertension nor biological hypercortisolism. One hundred patients were finally included in this study. Genetic analysis revealed five original heterozygous *NR3C1* mutations (see flow chart in Fig. 1).

Characteristics of all included patients are reported in Table 1. Among eligible patients, mean age was 64.2 ± 9.2 years with a sex ratio close to 1. More than eighty-seven percent of these patients had hypertension, most of them (79%) had an antihypertensive therapy. Patients had a high BMI (29.8 ± 6.2 kg/m$^2$). Thirty-four percent of the patients presented with diabetes. Twenty-two percent of the patients exhibited cardiac diseases (heart failure, coronary deficiency, coronary artery disease). Total cholesterol was in the normal range with normal HDL cholesterol and LDL cholesterol levels. Thirty-four patients had elevated UFC above ULN, while 40 patients had plasma cortisol levels >50 nmol/L after 1 mg DST. Fourteen patients had both abnormalities while among them, 9 patients had also normal or high ACTH levels and 5 patients had low ACTH levels (<10 pg/mL), suggesting biological autonomous hypercortisolism.

All patients had bilateral hyperplastic adrenal glands with high average volumes of right and left adrenals, measured at 10.2 and 13.4 cm$^3$, respectively. Hyperplasia was due to either lateral or medial limb >5 mm or adrenal adenomas >10 mm (Fig. 2) or right or left adrenocortical volumes above normal range (Table 1). Forty eight percent of patients had right adrenal hyperplasia while 75% had left adrenal hyperplasia, 71% had right nodular adrenal and 81% had left nodular adrenal.

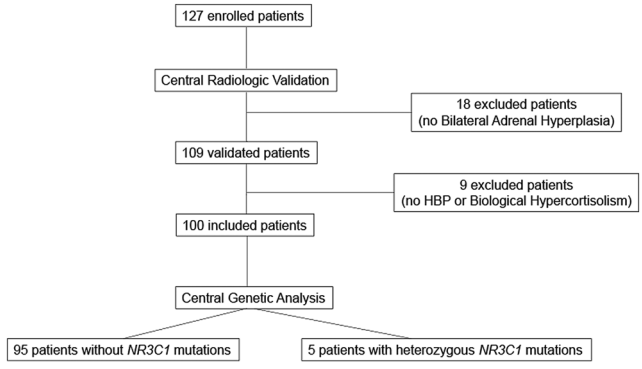


**Figure 1**
Flow chart of the study design. 127 patients were initially enrolled. After standardized examination of MDCT by an expert radiologist (CHU Reims), 18 patients were excluded because they had no bilateral adrenal incidentalomas. Nine patients had no clinical or biological inclusion criteria: neither hypertension (HBP) nor biological hypercortisolism. One hundred patients were finally included in the Muta-GR study. Centralized genetic analysis (CHU Bicêtre) identified 5 original heterozygous *NR3C1* mutations. HBP, high blood pressure; *NR3C1* gene encoding for the human glucocorticoid receptor (GR).



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**Table 1** Basal characteristics of 100 included patients.

| | *n* (data) | All patients | Normal range |
|---|---|---|---|
| Clinical | | | |
| Age (years) | 100 | 64.2±9.2 | |
| Sex M/W | 100 | 52 (52.0%)/48 (48.0%) | |
| Hypertension | 100 | 87 (87.0%) | |
| Antihypertensive treatment | 100 | 79 (79.0%) | |
| BMI (kg/m$^2$) | 98 | 29.8±6.2 | <25 |
| Waist circumference (cm): men | 15 | 104.7±13.7 | <94 |
| Waist circumference (cm): women | 15 | 100.7±19.6 | <80 |
| Diabetes | 100 | 34 (34.0%) | |
| Cardiac disease | 100 | 22 (22.0%) | |
| Biological | | | |
| Kalemia (mmol/L) | 95 | 4.1±0.5 | 3.5–5 |
| Fasting glycaemia (mmol/L) | 82 | 6.2±2.1 | <7.8 |
| HbA1c (%) | 68 | 6.5±1.2 | <6.5 |
| Total cholesterol (g/L) | 89 | 1.9±0.4 | <2 |
| HDL cholesterol (g/L) | 86 | 0.5±0.2 | >0.45 |
| LDL cholesterol (g/L) | 85 | 1.1±0.4 | <1 |
| Hormonology | | | |
| UFC (>N) | 93 | 0.9±0.8 N | <1 |
| 8-AM cortisol (nmol/L) | 94 | 448±218 | 248–635 |
| Midnight cortisol (nmol/L) | 64 | 160±145 | <50 |
| 8-AM ACTH (pg/mL) | 91 | 17.0±18.6 | 10–50 |
| Cortisol after 1 mg DXM test (nmol/L) | 65 | 104±126 | <50 |
| Radiology | | | |
| Right adrenal volume (cm$^3$) | 96 | 10.2±5.7 | 2.5–5.1 |
| Left adrenal volume (cm$^3$) | 94 | 13.4±6.7 | 2.9–6.1 |
| Total adrenal volume (cm$^3$) | 94 | 23.6±10.5 | 5.7–11.1 |
| Right adrenal hyperplasia | 100 | 48 (48.0%) | |
| Left adrenal hyperplasia | 100 | 78 (75.7%) | |
| Bilateral hyperplasia | 100 | 48 (48.0%) | |
| Bilateral nodular | 100 | 21 (21%) | |
| Left hyperplasia and right nodular | 100 | 20 (20.0%) | |
| Left hyperplasia and elevated right adrenal volume | 100 | 26 (26.0%) | |
| Right nodule >10 mm | 100 | 71 (71%) | |
| Left nodule >10 mm | 100 | 81 (81%) | |

Clinical, biochemical, hormonal, radiological features of the 100 included patients.
BMI, body mass index; DST, dexamethasone suppression test; HbA1C, glycated hemoglobin; HDL, high density level; LDL, low density level; M/W, men/women; UFC, 24-h urinary free cortisol; ULN, upper limit of normal range.

### Five heterozygous *NR3C1* mutations

Five original heterozygous *NR3C1* mutations were discovered in our series. As illustrated in Fig. 3, three patients harbored heterozygous missense mutations: R477S (arginine (R) to serine (S) substitution at amino-acid position 477) located in the exon 4 encoding for the DBD, Q501H (glutamine (Q) to histidine (H) substitution at amino-acid position 501 in the exon 5) and L672P

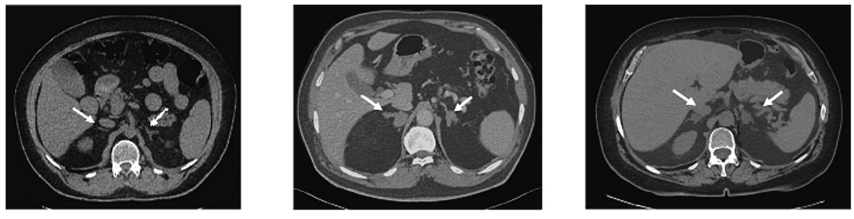


**Figure 2**
Radiological examples of adrenal bilateral hyperplasia. MDCT examinations. Left panel: normal adrenal glands. Bilateral adrenal hyperplasia (middle panel) and thin adrenal limb with bilateral macroadenoma (right panel) are presented. White arrows indicate the position of adrenal glands.


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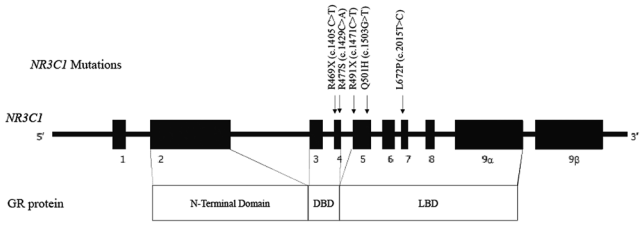


**Figure 3**

*NR3C1* mutations identified. Localization of the 5 heterozygous GR mutations on the *NR3C1* gene with schematic representation of the exon composition. Two mutations are located in the DBD, R469X (arginine (R) to stop (X) substitution at amino-acid position 469), R477S (arginine (R) to serine (S) substitution at amino-acid position 477) and 3 mutations are located in the ligand-binding domain (LBD), R491X (arginine (R) to stop (X) substitution at amino-acid position 491), Q501H (glutamine (Q) to histidine (H) substitution at amino-acid position 501), L672P (leucine (L) to proline (P) substitution at amino-acid position 672).

(leucine (L) to proline (P) substitution at amino-acid position 672 in the exon 7) located in the LBD. Two patients carried heterozygous nonsense mutations: R469X (arginine (R) to stop (X) substitution at amino-acid position 469 in the exon 4) located in the DBD and R491X (arginine (R) to stop (X) substitution at amino-acid position 491 in the exon 5), located in the LBD. Functional *in vitro* characterization of R469X, R477S and L672P have already been reported and revealed impairment in GR signaling for all GR mutants (16, 27). Collectively, this indicates a global prevalence of GR mutations of 5% among 100 included patients.

The clinical, biological, hormonal and radiological characteristics of these mutated patients were compared to those of non-mutated patients (Table 2). Mutated patients were younger (53.2±7.7 years old, means±S.D., $P=0.007$), but only 2 mutated patients out of five (40%) had hypertension compared to 89.5% of the non-mutated patients ($P=0.02$). The percentage of cardiovascular events was not different between non-mutated and mutated patients, likewise for cardiac risk factors (diabetic features, BMI and LDL cholesterol), except for HDL cholesterol, slightly higher (0.70±0.27 g/L) in mutated patients compared to non-mutated patients (0.51±0.17 g/L, $P=0.03$). As expected, higher UFC was found in mutated patients compared to the non-mutated patients (1.7±0.7 vs 0.9±0.8 ULN, $P=0.006$, respectively) without suppressed 8-AM ACTH levels (30.9±31.2 pg/mL in mutated patients vs 16.2±17.5 pg/mL), associated with more elevated cortisol after 1 mg DST (227.4±174 vs 93.4±117.4 nmol/L, $P=0.035$). There was no difference for midnight plasma cortisol between mutated and non-mutated patients. Among the 5 mutated patients, 4 patients had an increased UFC, normal or high ACTH levels and elevated cortisol after 1 mg DST, consistent with glucocorticoid resistance syndrome.

Potassium levels were lower in mutated patients compared to non-mutated patients (3.6±0.2 vs 4.1±0.5 mmol/L, $P=0.01$). Interestingly, aldosterone levels were statistically lower in mutated patients (17.3±9.9 vs 98.6±115.4 pg/mL in non-mutated patients, $P=0.0011$). Renin levels were low or normal in all mutated patients except for the patient harboring R477S mutation due to angiotensin-converting enzyme (ACE) inhibitor treatment. Therefore, low plasma renin and aldosterone levels, associated with kalemia <4 mmol/L are recurrent biological features prevailing in mutated patients of the Muta-GR cohort.

Adrenocortical morphology of mutated patients (number of nodules, adrenal hyperplasia, TAV) was not different from non-mutated patients. One patient had bilateral adrenal hyperplasia (patient with R469X mutation), one had thin bilateral nodular adrenals (patient with L672P mutation) and others had either nodular thin or right/left adrenal hyperplasia.

## GR haploinsufficiency in mutated patients

We obtained fibroblasts from skin biopsies of four mutated patients (patients carrying GR R469X, R477S, R491X, Q501H mutations, since patient with GR L672P mutation was lost of follow-up). We confirmed by sequencing that these skin fibroblasts carried both normal and mutated GR alleles and thus presumably endogenously expressed both wild-type and mutated GR, notwithstanding the possibility of nonsense mediated RNA decay, previously demonstrated for GR stop mutation (16). We next evaluated directly the DXM induction of a well-known glucocorticoid-induced target gene *FKBP5* in fibroblasts' patients (Fig. 4).

After 6-h DXM exposure, expression levels of FKBP5 mRNA were significantly lower in all mutated fibroblasts ($GR^{WT/mut}$) compared to non-mutated fibroblasts ($GR^{WT/WT}$ (control): 50% for $GR^{WT/R469X}$ (R469X) ($P<0.01$), 22.7% for $GR^{WT/R477S}$ (R477S) ($P=0.0018$), 31.3% for $GR^{WT/R491X}$ (R491X) ($P=0.0101$), 1.3% for $GR^{WT/Q501H}$ (Q501H) ($P=0.0022$)). These findings provide direct support for a GR haploinsufficiency, driven by heterozygous *NR3C1* mutations, consistent with glucocorticoid resistance observed in the mutated patients.





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**Table 2** Clinical, biochemical, hormonal and radiologic characteristics of the non-mutated vs mutated patients.

| | *n* (data) | Non-mutated patients | *n* (data) | Mutated patients | *P* | R469X | R477S | R491X | Q501H | L672P |
|---|---|---|---|---|---|---|---|---|---|---|
| Clinical | | | | | | | | | | |
| Age (years) | 95 | 64.7±8.9 | 5 | 53.2±7.7 | **0.007** | 56 | 50 | 44 | 60 | 46 |
| Sex M/W | 95 | 49.4% (46)/51.6% (49) | 5 | 40% (2)/60% (3) | NS | M | W | M | W | M |
| Hypertension | 95 | 89.5% (85) | 5 | 40% | **0.02** | YES | YES | NO | NO | NO |
| Diabetes | 95 | 35% (33) | 5 | 20% | 0.66 | NO | YES | NO | NO | NO |
| Cardiac disease | 95 | 22% (21) | 5 | 20% | NS | YES | NO | NO | NO | NO |
| BMI (kg/m$^2$) | 93 | 29.8±6.0 | 5 | 30.7±9.5 | 0.83 | 26 | 46.9 | 31.1 | 23.7 | 25.8 |
| Biology | | | | | | | | | | |
| Kalemia (mmol/L) | 90 | 4.1±0.5 | 5 | 3.6±0.2 | **0.01** | 3.5 | 3.5 | 3.8 | 3.8 | 3.6 |
| Fasting glycaemia (mmol/L) | 77 | 6.2±2.2 | 5 | 5.4±0.7 | 0.5 | 4.8 | 5.1 | 5.5 | 6.5 | 5 |
| HbAIC (%) | 65 | 6.5±1.2 | 3 | 5.6±0.5 | 0.17 | 5 | 5.9 | 5.9 | | |
| Total cholesterol (g/L) | 84 | 1.85±0.44 | 5 | 1.97±0.61 | 0.5 | 1.2 | 1.5 | 2.25 | 2.2 | 2.7 |
| HDL cholesterol (g/L) | 82 | 0.51±0.17 | 4 | 0.70±0.27 | **0.03** | 0.6 | 0.6 | 0.52 | | 1.1 |
| LDL cholesterol (g/L) | 81 | 1.1±0.4 | 4 | 1.1±0.6 | 0.92 | 0.5 | 0.6 | 1.62 | | 1.6 |
| Hormonology | | | | | | | | | | |
| UFC (ULN) | 88 | 0.9±0.8 | 5 | 1.7±0.65 | **0.006** | 1.74 | 1.66 | 2 | 0.68 | 2.46 |
| 8-AM cortisol (nmol/L) | 89 | 440±216 | 5 | 614±213 | 0.1 | 587 | 896 | 552 | 320 | 715 |
| Midnight cortisol (nmol/L) | 59 | 160±150 | 5 | 165±73 | 0.35 | 119 | 113 | 240 | 103 | 248 |
| 8-AM ACTH (pg/mL) | 86 | 16.2±17.5 | 5 | 30.9±31.2 | 0.16 | 11.5 | 26 | 85 | 8 | 24 |
| Cortisol >50 nmol/L after 1 mg DST (nmol/L) | 60 | 93.4±117.4 | 5 | 227.4±174.0 | **0.035** | 119 | 164 | 463 | 41 | 350 |
| Renin (pg/mL) | 67 | 33.0±82.2 | 5 | 47.2±92.6 | 0.65 | 2.5 | 212.7 | 2 | 7 | 12 |
| Aldosterone (pg/mL) | 70 | 98.6±115.4 | 5 | 17.3±9.9 | **0.0011** | 22 | 16.6 | 20 | <27 | 1 |
| Radiology | | | | | | | | | | |
| Right adrenal volume (cm$^3$) | 91 | 10.5±5.8 | 5 | 8.8±3.3 | 0.57 | 12.6 | 6.1 | 7.1 | 5.9 | 12.2 |
| Left adrenal volume (cm$^3$) | 89 | 13.6±6.8 | 5 | 12.9±6.9 | 0.9 | 16.8 | 9.1 | 10.1 | 5.1 | 22.7 |
| Total adrenal volume (cm$^3$) | 89 | 24.0±10.6 | 5 | 21.7±10.1 | 0.63 | 29.4 | 15.2 | 17.9 | 11 | 34.9 |
| Right adrenal hyperplasia | 92 | 51.0% (47) | 5 | 20% (1) | 0.36 | YES | NO | NO | NO | NO |
| Left adrenal hyperplasia | 93 | 76.5% (71) | 5 | 80% (4) | NS | YES | YES | YES | YES | NO |
| Right nodule >10 mm | 95 | 71.6% (68) | 5 | 60% (4) | 0.63 | YES | NO | YES | NO | YES |
| Left nodule >10 mm | 95 | 81.0% (77) | 5 | 80% (4) | NS | YES | NO | YES | YES | YES |

BMI, body mass index; DST, dexamethasone suppression test; HbA1C, glycated hemoglobin; HDL, high density level; LDL, low density level; M/W, men/women; UFC, 24-h urinary free cortisol; ULN, upper limit of normal range.



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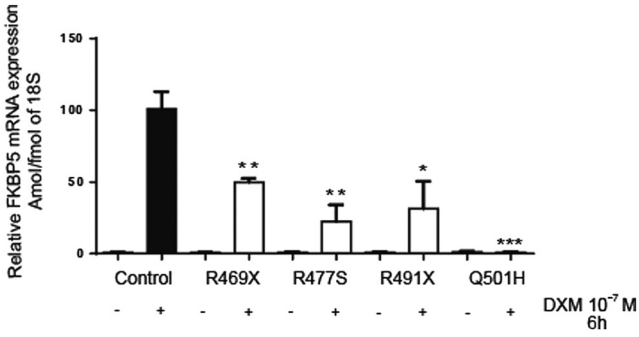


**Figure 4**

GR haploinsufficiency in mutated patients' fibroblasts. *Ex vivo* characterization of GR mutations in patients' fibroblasts. Patient with L672P mutation was lost of follow-up. *FKBP5* is a GR target gene. After 6-h exposure of vehicle or $10^{-7}$ M DXM, *FKBP5* mRNA expression was measured by RT-qPCR in fibroblasts and normalized with rRNA 18S. The DXM induction of *FKBP5* mRNA in patient's fibroblasts with wild-type GR (Control) was arbitrarily set at 100%. Results are mean ± S.E.M. of two independent determinations performed in duplicate. $*P<0.05$, $**P<0.01$, $***P<0.005$ was statistically significant with non-parametric Mann–Whitney *U* tests.

### GR polymorphisms

We also evaluated some common GR polymorphisms frequently described in the general population, notably ER22/23EK (rs6189 and rs6190, with minor allele frequency of 1.8% and 1.8%, respectively), rs10482622 (0.8% in the general population), N363S (rs6195, 2.1%), all located in the exon 2 and *BclI* (rs41423247, 25.5%), located in the intron 2, rs6188 located in the intron 4 and pN766N (rs6196) located in the 9α exon (26.4% and 13.0%, respectively) (Fig. 5). Minor allelic frequencies of rs6189 and rs6190 (ER22/23EK), rs10482622, N363S, rs6188 and pN766N were calculated at 1.98, 1.98, 0.50, 0.50, 22.8 and 14.8, respectively, in the patients included in Muta-GR study (Table 3), without any difference with the general population as estimated by the exome aggregation consortium (ExaC: https://www.exac.broadinstitute.org/ and 1000 genomes: http://www.internationalgenome.org/). Only *BclI* polymorphism (rs41423247) was over-represented in this cohort (38.7% vs 25.5% in the general population, $P=0.0045$). Only one mutated patient had a heterozygous polymorphism rs6188.

### Selected criteria to search for *NR3C1* mutations in eligible patients

In order to select patients with bilateral adrenal hyperplasia suitable for GR molecular investigation,

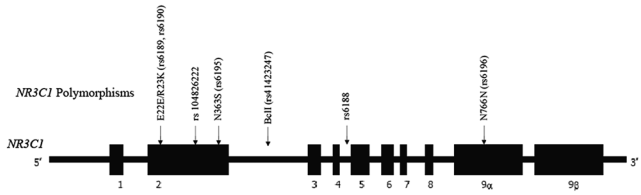


**Figure 5**

GR polymorphisms of 100 included patients. Localization of GR polymorphisms: ER22/23EK (rs6189, rs6190), rs10482622, N363S (rs6195) located in the exon 2, *BclI* (rs41423247) located in the intron 2, rs6188 located in the intron 4, N766N (rs6196) located in the exon 9α.

we searched for the common clinical and/or biological features of mutated patients. Clinically, as expected, none of them had any Cushing's syndrome signs. Four out of 5 mutated patients had only mild glucocorticoid resistance syndrome. However, all mutated patients presented with kalemia <4.0 mmol/L contrasting with plasma aldosterone levels below the lower limit of normal values. Finally, as depicted in Fig. 6, among 100 included patients in the Muta-GR study, the initial prevalence of GR mutations evaluated at 5% (5/100 patients), increased up to 16% (5/32 patients) in those with bilateral adrenal hyperplasia AND low or normal aldosterone levels AND kalemia <4 mmol/L. Of importance, the prevalence of GR mutations increased up to 44% (4/9 patients) if one takes into account the four following criteria: Bilateral adrenal hyperplasia and glucocorticoid resistance syndrome (elevated UFC or elevated 8.00 AM cortisol after 1 mg DST) and low aldosterone levels and kalemia <4 mmol/L.

## Discussion

We identify 5 heterozygous GR mutations in patients presenting with bilateral adrenal hyperplasia associated with biological hypercortisolism or hypertension without Cushing's syndrome signs among 100 included patients in the Muta-GR study. Therefore, the prevalence of *NR3C1* mutations in the Muta-GR patients is 5%. Up to date, only 26 GR loss-of-function mutations (including ours) have been reported in the literature, which leads us to believe that the prevalence of *NR3C1* mutations is largely underestimated.

Historically, the first GR mutation was described in a patient with severe hypertension and hypokalemia associated with a glucocorticoid resistance syndrome characterized by high UFC and high 8-AM ACTH level, consistent with the lack of negative feedback of cortisol



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**Table 3** GR polymorphisms.

| SNP polymorphism | Nucleotides | Protein | *n* (data) | ExaC or 1000 genomes | *P* |
|---|---|---|---|---|---|
| rs6189 | c.66G>A | E22E | | | |
| GG | | | 95 | | |
| GA | | | 5 | | |
| Carrier of allele A | | | 1.98% | 1.8% (2216/126280) | 0.64 |
| rs6190 | c.68G>A | R23K | | | |
| GG | | | 96 | | |
| GA | | | 4 | | |
| Carrier of allele A | | | 1.98% | 1.8% (2216/126280) | 0.62 |
| rs10482622 | c.879G>A | K293K | | | |
| GG | | | 99 | | |
| GA | | | 1 | | |
| Carrier of allele A | | | 0.50% | 0.8% (941/121252) | 0.96 |
| N363S (rs6195) | c.1088A>G | N363S | | | |
| AA | | | 99 | | |
| AG | | | 1 | | |
| Carrier of allele G | | | 0.50% | 2.1% (2163/121268) | 0.2 |
| *BclI* (rs41423247) | c.1184+646C>G | | | | |
| CC | | | 35 | | |
| CG | | | 44 | | |
| GG | | | 14 | | |
| Carrier of allele G | | | 38.70% | 25.5% (1275/5008) | **0.0045** |
| rs6188 | c.1469-16G>T | | | | |
| GG | | | 61 | | |
| GT | | | 36 | | |
| TT | | | 3 | | |
| Carrier of allele T | | | 22.80% | 26.4% (31472/119366) | 0.16 |
| rs6196 | c.2298A>G | pN766N | | | |
| AA | | | 73 | | |
| AG | | | 24 | | |
| GG | | | 3 | | |
| Carrier of allele G | | | 14.80% | 13% (15723/121026) | 0.56 |

ER22/23EK polymorphism corresponds to two linked polymorphisms in codons 22 and 23 GGA.GAG to GAA.AAG leading to one amino acid change ER to EK (4). The rs10482622 is a T→C: 12 patients were heterozygous (TC) and 3 patients homozygous (CC) for this polymorphism. N363S is the result of a A→G change. One patient carries this polymorphism. *BclI* polymorphism (rs41423247) is a C to G change. 38.7% of Muta-GR patients carry this polymorphism. The rs6188 polymorphism is the result of the changing of G→T. Thirty-six patients were heterozygous (GT) and 3 patients homozygous (TT). pN766N (rs6196) is the result of an A→G substitution: 24 patients were heterozygous (AG) and 3 were homozygous (GG).

on HPA axis (33, 34). Likewise, patients carrying the thirteen GR loss-of-function mutations, subsequently described up to 2010 (6, 7, 8, 10, 12, 13, 15), exhibited an overt glucocorticoid resistance phenotype. Obviously, our present study underscores the large variety of clinical, biological and radiological signs that could be associated with GR mutations. One patient (Q501H mutation carrier) had only an elevated midnight plasma cortisol

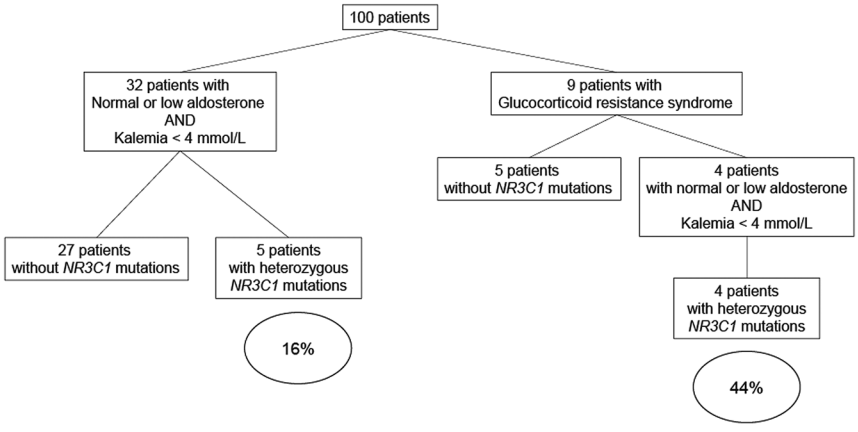


**Figure 6**
High prevalence of *NR3C1* mutations in selected patients. Among 100 included patients, 32 patients had normal or low aldosterone levels associated with kalemia <4 mmol/L resulting in a prevalence of GR mutations increased to 16%. Nine patients also had glucocorticoid resistance syndrome, leading to the prevalence of GR mutations increased up to 44% in such patients.





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level but with preservation of the negative feedback loop on HPA axis. Although increased ACTH levels have been regularly reported in some cases as a direct consequence of glucocorticoid resistance, this does not seem to be the case for 4 out of 5 patients of our study since only one patient had high 8-AM ACTH level (R491X mutation carrier). However, the ACTH level values, not evaluated within the very same center, should be interpreted with an extreme caution given the unstable nature of this hormonal analyte (35) and the absence of reliable and standardized measurements (36). Altogether, our data indicate that the generalized glucocorticoid resistance syndrome seems to be relatively mild in patients harboring GR loss-of-function mutations, compared to the first cases described to date. The main differential diagnosis of mild glucocorticoid resistance could be the pseudo-Cushing syndrome, whose diagnostic relies on moderately elevated UFC (below twice the ULN) and partial failure of 1 mg DST in obese, poorly controlled diabetic, depressed or alcoholic patients (37). Four out of five mutated patients, reported in the present study, presented with these clinical and biochemical features suggesting that *NR3C1* sequencing should be performed in some atypical pseudo-Cushing patients to comfort glucocorticoid resistance.

Furthermore, all mutated patients in the Muta-GR study showed alteration in the mineralocorticoid pathway. Indeed, low aldosterone levels with normal or low renin levels with or without hypertension sharply contrast with low plasma potassium levels. The first glucocorticoid-resistant patient described in 1976 had hypertension with hypokalemia, which was interpreted as a direct consequence of increased plasma deoxycorticosterone (DOC) and corticosterone concentrations due to overstimulation of adrenal glands by ACTH hypersecretion (33, 34). Interestingly, DOC levels were in the normal range in the 3 patients in whom these were measured (R469X (16), R477S (27) and R491X) carrier patients. Low urinary tetrahydrocortisone (THE)/tetrahydrocortisol (THF) ratio were found in all affected GR 469X carriers, suggesting an impairment of renal 11-β hydroxysteroid dehydrogenase type 2 (11-βHSD2) activity (16). Indeed, 11-βHSD2 enzyme metabolizes cortisol into cortisone, thus preventing cortisol to bind and activate the renal mineralocorticoid receptor (MR), responsible for sodium retention, volume expansion and HBP. Few cases of 11-βHSD2 deficiency have been described in a context of ectopic ACTH syndrome (38) and *HSD11B2* gene mutations (39). Moreover, *HSD11B2* gene seems to be a glucocorticoid-inducible target gene since human placental 11-βHSD2 mRNA expression increased after DXM stimulation that was inhibited by GR antagonist RU486 (40), suggesting the involvement of GR in the regulation of 11-βHSD2 activity. Taken together, we propose that altered GR signaling may be responsible for a decreased 11-βHSD2 activity, leading to inefficient glucocorticoid breakdown. As a result, elevated cortisol concentrations in mutated patients could induce illicit occupation and activation of the unprotected MR which, in turn, could lead to hypokalemia despite low plasma aldosterone levels, in the context of apparent mineralocorticoid excess or pseudohypermineralocorticism.

Herein, we demonstrate GR haploinsufficiency in mutated patients and propose that it could be a potential cause of bilateral adrenal hyperplasia. Indeed, several authors already demonstrated the expression of GR in the normal adrenal gland (41) and its involvement in different pathologies such as adrenocortical carcinomas (42) or nodules of primary pigmented nodular adrenocortical disease tissues (43).

One recurrent pathophysiological mechanism evoked for bilateral adrenal hyperplasia in the context of GR loss-of-function mutations was the ACTH overstimulation of the adrenal glands. This was comforted by various human and mouse studies. A single case of bilateral adrenal hyperplasia was reported in a 7-year-old boy with high ACTH level associated with heterozygous GR mutation (13). Invalidation of the *Nr3c1* gene in mice was accompanied by an enlarged size of adrenal glands and disorganized adrenocortical cells (44). As expected, although most of $GR^{-/-}$ mice died 1–2 h postnatally, they presented with high corticosterone and ACTH levels consistent with altered HPA regulation. However, heterozygous $GR^{+/-}$ mice also had larger adrenal glands (17) but normal ACTH levels, precluding an exclusive role of high circulating ACTH levels in the pathogenesis of adrenal hyperplasia. In our study, the majority of mutated patients had normal ACTH levels with no hirsutism in women and no elevated plasma DOC levels, ruling out a simple ACTH overstimulation of the adrenal glands. Along this line, a positive ultrashort regulatory loop exerted by glucocorticoids via GR on steroidogenesis was reported in human adrenocortical H295R cells, independently of ACTH signaling (45). Collectively, the mechanisms by which GR haploinsufficiency may lead to bilateral adrenal hyperplasia remain to be elucidated but clearly cannot be simply restricted to a chronic ACTH overstimulation, even though we have not explored the potential role of intra-adrenal endogenous ACTH release, recently reported (46).

The second objective of the Muta-GR study was to evaluate *NR3C1* polymorphism frequencies associated





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with adrenal hyperplasia compared to the general population. *BclI* polymorphism (rs41423247) was over-represented in the Muta-GR cohort compared to the general population, in contrast to Tzanela *et al.* (47), who did not detect higher frequency of bilateral masses in *BclI* carriers among 95 patients with adrenal incidentalomas. Nevertheless, despite the number of Muta-GR patients, a direct relationship between bilateral adrenal incidentaloma and minor allelic frequencies of some common GR polymorphisms (rs6189, rs6190, rs10482622, rs6195, rs41423247, rs6188 and rs6196) has not been established. This contrasts with other studies that have suggested a role of these polymorphisms in unilateral adrenal incidentaloma (47, 48). Altogether, except *BclI*, no association was found between the presence of such polymorphisms and bilateral adrenal hyperplasia in our series.

There are several limitations of this multicenter study. Indeed, MDCT examinations were performed in each independent center on different MDCT units and with various protocols of image acquisitions so that the axial section thickness was different among the centers and the measures of adrenal volume could have been slightly impacted by these differences (29). However, analysis of adrenal hyperplasia's appearance with manual measurements of adrenal limbs and bodies, as well as assessment of the presence of nodules and calculation of adrenal glands volume was centralized and performed by a single operator. Moreover, biochemical and hormonal analyses were performed in each center with inherent methodological differences and variations in normal ranges, impeding direct value comparison especially for some specific hormones as mentioned earlier. This led us to express some results such as UFC, normalized to the ULN.

Our study has direct clinical implications. Indeed, identification of *NR3C1* mutation in a patient presenting with adrenal hyperplasia and alteration of cortisol secretion excludes false diagnosis of preclinical Cushing's syndrome or pseudo-Cushing's syndrome. Molecular screening could be extended to the direct family members. This has also important consequences on the follow-up and management of such patients, in whom no further invasive test should be proposed, adrenocortical surgery should be excluded while appropriate pharmacological strategies (mineralocorticoid antagonists) could be envisioned. The pathophysiological significance and the precise clinical pathogenesis of *NR3C1* mutations remain to be clearly established. However, one has to take into account the presence of pseudohypermineralocorticism associated with these genetic alterations, especially with respect to its potential impact on cardiovascular system.

In conclusion, we discovered 5 original heterozygous GR mutations in a population of 100 patients with bilateral adrenal hyperplasia associated with biological hypercortisolism and/or hypertension without Cushing's syndrome signs. Most of mutated patients harbored a mild generalized glucocorticoid resistance and a pseudohypermineralocorticism. We suggest that *NR3C1* genetic alterations should be systematically searched in selected patients notably those presenting with eligible criteria, as well as in pseudo-Cushing patients for the benefit of both the patients and their families.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

**Funding**

G V is the recipient of a doctoral fellowship from the Fondation pour la Recherche Médicale (FRM). This work was supported by the French Ministry of Health (Programme Hospitalier de Recherche Clinique National) (PHRC-2011-N11-01) and grants from Institut National de la Santé et de la Recherche Médicale (Inserm) and Paris Sud University.

**Author contribution statement**

B D and M L contributed to the study concept and design. Investigators referred the patients. G V performed the analysis. C H supervised C T examination. G V performed statistical analysis. J B analyzed G R mutations and polymorphisms. G V, S T, C H, B D and M L contributed to the drafting of the paper and shared the responsibility to submit the manuscript for publication. All authors analyzed the data, corrected and approved the final version of the manuscript.

**Acknowledgments**

List of investigaors in the MUTA-GR study: Dr F Amiot-Chapoutot (CH Troyes, France); Dr D Ancelle (CH St Lô, France); Dr F Bertoin (CHU Reims, France); Prof. T Brue (CHU Marseille, France); Prof. P Caron (CHU Toulouse, France); Prof. F Borson-Chazot (HCL, Lyon, France); Prof. S Christin-Maitre (CHU St Antoine, Paris, France); Prof. O Chabre (CHU Grenoble, France); Prof. R Dessailloud (CHU Amiens, France); Prof. B Estour (CHU Saint-Etienne, France); Dr H Grulet (CHU Reims, France); Dr F Illouz (CHU Angers, France); Prof. N Jeandidier (CHU Strasbourg, France); Prof. V Kerlan (CHU Brest, France); Prof. M Klein (CHU Nancy, France); Prof. A Penfornis (CHU Besançon, France); Dr P Pierre (CHU Tours, France); Prof. A Tabarin (CHU Bordeaux, Pessac, France); Prof. P Touraine (CHU Pitié-Salpetrière, Paris, France); Prof. M C Vantyghem (CHRU Lille, France); Prof. J Young (CHU Bicêtre, Le Kremlin Bicêtre, France).

The authors would like to thank Dr Coralie Barbe and Edith Kassa (CHU Reims) who helped us to manage the Muta-GR study. They thank Isabelle Boucly (CHU Bicêtre) for excellent technical assistance in GR mutation screening and Neila Belassaoui (CHU Bicêtre) for GR polymorphism determination. They also are indebted to Dr Alexandre Buffet (CHU Toulouse) for providing us with the skin biopsy of one patient and Dr Eric Pussard (CHU Bicêtre) for LCMSMS measurements of steroidomic profiling in one patient. G V is the recipient of a doctoral fellowship from the 'Fondation pour la Recherche Médicale' (FRM).



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Received 26 December 2017
Revised version received 9 February 2018
Accepted 14 February 2018



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