Received: 4 October 2018 | Revised: 3 March 2019 | Accepted: 13 May 2019

DOI: 10.1111/eci.13129

ORIGINAL ARTICLE

WILEY

Transcriptomics in tissue glucocorticoid sensitivity

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Abstract

Background: Synthetic glucocorticoids are widely used in the treatment of several inflammatory, autoimmune and lymphoproliferative disorders. However, considerable variation in response to therapeutic doses of glucocorticoids has been documented among individuals. The aim of our study was to identify novel glucocorticoid sensitivity-determining genes using genome-wide expression profiling in healthy subjects.

Methods: One hundred one healthy subjects [mean age ± standard error of the mean (SEM); 26.52 ± 0.50 years] were given 0.25 mg dexamethasone at midnight, and serum cortisol concentrations were determined at 08:00 hours the following morning. Subjects were stratified into the 10% most glucocorticoid-sensitive and 10% most glucocorticoid-resistant according to the serum cortisol concentrations. Genomic DNA, RNA and plasma samples were obtained in the 22 subjects one month later.

Results: Transcriptomic analysis showed variability between glucocorticoid-resistant and glucocorticoid-sensitive subjects. One hundred thirty-three genes were upregulated and 49 downregulated in the glucocorticoid-resistant compared to the glucocorticoid-sensitive group. Further analysis revealed differences between 3 glucocorticoid-resistant and 3 glucocorticoid-sensitive subjects. The majority of the 1058 upregulated genes and 1139 downregulated genes were found to participate in telomere maintenance, systemic lupus erythematosus and Alzheimer's disease. Interestingly, Synuclein A, a key molecule in Parkinson's disease, was upregulated in the subgroup of glucocorticoid-sensitive subjects.

Conclusions: We have identified differences in tissue sensitivity to glucocorticoids among healthy subjects at the transcriptomic level. These differences are associated with differential expression of genes related to autoimmune and neurological disorders.

KEYWORDS

glucocorticoid receptor, glucocorticoids, RNA sequencing, telomere length, tissue glucocorticoid sensitivity, transcriptomics

1 | INTRODUCTION

Glucocorticoids play an important role in several biologic functions and contribute substantially to the maintenance of basal and stress-related homeostasis.[1-3] These steroid hormones exert potent anti-inflammatory and immune-modulating genomic and nongenomic effects through their cognate receptor, the human glucocorticoid receptor (hGR), which is a member of the steroid receptor family of transcription factors.[4] In addition, synthetic glucocorticoid analogues have been used worldwide in the therapeutic management of inflammatory, autoimmune and lymphoproliferative disorders.[5] However, patients have shown a considerable variation in response to therapeutic doses of glucocorticoids in terms of disease progression and side effects.[6-13]

Accumulating evidence suggests that tissue sensitivity to glucocorticoids is influenced by several factors participating in the signalling cascade of glucocorticoids. Indeed, genetic factors, such as polymorphisms, mutations, deletions and/or insertions in the *NR3C1* gene, have been associated with glucocorticoid resistance or hypersensitivity syndromes.[13,14] Carriers of the ER22/23EK exhibit a glucocorticoid-resistant phenotype characterized by improved body composition, healthier metabolic profile, prolonged survival and lower risk of dementia. On the other hand, individuals harbouring the N363S or *Bcl1* polymorphisms present with clinical manifestations of glucocorticoid hypersensitivity, such as increased weight gain, insulin resistance and metabolic complications.[13,14] In addition to the above-discussed polymorphisms, an ever-increasing number of *NR3C1* mutations, insertions or deletions have been shown to impair the glucocorticoid signal transduction pathway.[15] The majority of them have been associated with primary generalized glucocorticoid resistance syndrome, whereas only one *NR3C1* mutation, the hGRαD401H, has been identified in a patient with glucocorticoid hypersensitivity.[15,16] In addition to the *NR3C1* polymorphisms or genetic defects, several other factors, including hGR isoforms and post-translational modifications, as well as RNA molecules (miRNA, noncoding RNA), influence tissue sensitivity to glucocorticoids.[17-20]

Although several molecular and cellular factors have been shown to modulate glucocorticoid sensitivity, a small number of studies have investigated the gene expression profile that might predict tissue glucocorticoid response.[21,22] The aim of this study was to identify novel genes determining tissue sensitivity to glucocorticoids using genome-wide expression profiling in healthy subjects.

2 | MATERIALS AND METHODS

2.1 | Subjects

One hundred one healthy subjects (mean age ± SEM; 26.52 ± 0.50 years), who had not previously received glucocorticoid treatment, were recruited to participate in this study. The study was approved by the "Aghia Sophia" Children's Hospital Committee on the Ethics of Human Research, and written informed consent was obtained by all participants. Subjects were given a low-dose (0.25 mg) dexamethasone at midnight, and serum cortisol and plasma ACTH concentrations were determined at 08:00 hours the following morning. Subjects were then polarized into the 10% most glucocorticoid-sensitive (n = 11) and 10% most glucocorticoid-resistant (n = 11) according to serum cortisol concentrations [(mean serum cortisol concentrations ± SEM; 1.25 ± 0.16 μg/dL in the glucocorticoid-sensitive group *vs* 22.56 ± 1.02 μg/dL in the glucocorticoid-resistant group, $P < 0.001$) (mean plasma ACTH concentrations ± SEM; 2.75 ± 0.73 pg/mL in the glucocorticoid-sensitive group *vs* 31.57 ± 3.19 pg/mL in the glucocorticoid-resistant group, $P < 0.001$)]. One month later, all subjects attended our outpatient clinic and blood samples for baseline haematological and endocrine investigations, as well as for DNA and RNA extraction, were drawn at 08:00 hours. Samples were centrifuged and separated immediately after collection, and were stored at −80°C until assayed.

2.2 | Sequencing of the *NR3C1* gene

Genomic DNA was isolated from peripheral blood leukocytes using the Maxwell 16 instrument for automated DNA extraction (Promega Corp.) as previously described.[23] The protein-coding sequences and the intron-exon junctions of the *NR3C1* gene were PCR-amplified and sequenced by using the Big Dye Terminator cycle sequencing kit (Applied Biosystems) on an ABI 3100 sequencer (ABI 3100, Applied Biosystems).

2.3 | Measurement of leukocyte telomere length (LTL)

Leukocyte telomere length (LTL) was measured using multiplex monochrome quantitative real-time PCR. All PCR reactions were performed in 96-well plates on a CFX real-time PCR detection system (Bio-Rad Laboratories). For each plate, two standard curves were generated: (a) the telomere amplicon signal (T) and (b) the single-copy gene (haemoglobin) amplicon signal (S). Telomere length was calculated by the ratio of telomere amplicon signal to single-copy gene control amplicon signal (T/S).[24]

2.4 | RNA sequencing

RNA-seq libraries were prepared using the TruSeq RNA kit using 1 μg of total RNA. The libraries were constructed according to Illumina's protocols and then were mixed in equal amounts. Single-end 75 bp reads for 22 patients were generated with NextSeq500 in the GGC. RNA-seq raw sequencing data were aligned to human genome version GCCh37/hg19

with the use of tophat (version 2.0.9)[25] with the use of «--b2-very-sensitive» parameter. Samtools (version 0.1.19)[26] were used for data filtering, and file format conversion while HT-seq count (version 0.6.1p1) algorithm[27] was performed for assigning aligned reads into exons using the following command line «htseq-count –s no –m intersection -nonempty». Differentially expression between groups was performed with the use of DESeq R package.[27] Genes with fold change cut-off ###1.5### and *P*-value ≤0.05 were considered to be differentially expressed (DEGs).

2.5 | Bioinformatics analysis

Gene ontology and pathway analysis were performed in the DEGs with DAVID knowledgebase[28] and Ingenuity Pathway Analysis software (IPA). Only pathways and biological processes with *P*-value ≤0.05 were considered to be significantly enriched. RNAseq data have been deposited in the Short Read Archive (SRA) under the accession codes SRPXXXX.

2.6 | Assays

The standard haematological investigations were determined using the ADVIA 2110i analyzer (Roche Diagnostics, GmbH).

Insulin, FSH, LH, oestradiol and ferritin concentrations were measured using automated electrochemiluminescence immunoassays "ECLIA" [Analyzer Cobas e411-ROCHE DIAGNOSTICS (GmbH)], while TSH, free T4, anti-TPO, anti-TG, testosterone, DHEA-S, androstenedione, cortisol, ACTH, IGF-I, IGFBP-3 and high sensitivity C-reactive protein (hs-CRP) concentrations were measured using automated chemiluminescence immunoassays on an IMMULITE 2000 Immunoassay System (Siemens Healthcare Diagnostics Products Ltd). Total 25-hydroxyvitamin D (25-OH Vitamin D) was measured using automated electrochemiluminescence immunoassay on the Modular Analytics E170 analyzer. HbA1C was determined using reversed-phase cation exchange high-performance liquid chromatography (HPLC) on an automated glycohemoglobin analyzer HA-8160 (Arkray).

2.7 | Statistical analyses

Data were analysed using the SPSS statistical package version 24.0 (SPSS). The continuous variables were summarized with the use of descriptive statistical measures and presented as mean ± standard error of the mean (SEM). The normality of distribution of continuous variables was examined using graphical methods (ie, histograms and Q-Q plots) in order to determine whether or not to use parametric methods for the analysis of the sample data. The associations between variables and groups of participants were evaluated by Student's *t* test or the Mann-Whitney *U* test for independent samples. All the aforementioned statistical tests were two-sided and performed at a 0.05 significance level.

3 | RESULTS

3.1 | Clinical characteristics and haematological and endocrinological data

One hundred one healthy subjects (50 males and 51 females) of a mean age (±SEM) 26.52 (±0.50) years were participated in the present study. Serum cortisol (9.49 ± 0.74 μg/dL) and plasma ACTH (16.10 ± 1.24 pg/mL) concentrations were determined at 08:00 hours in all participants following a low-dose dexamethasone. Participants were then polarized into the 10% most glucocorticoid-sensitive (n = 11) and 10% most glucocorticoid-resistant (n = 11) according to serum cortisol concentrations. Table 1 shows the haematological and endocrinological data of the 22 subjects. There was no statistically significant difference in the haematological and endocrinological parameters between the two groups [glucocorticoid-sensitive *vs.* glucocorticoid-resistant group ($P > 0.05$ for all)].

3.2 | Sequencing of the *NR3C1* gene revealed no genetic defects

To investigate whether this variation in tissue sensitivity to glucocorticoids could be attributed to genetic defects in the *NR3C1* gene, the protein-coding region and the intron/exon junctions were PCR-amplified and bidirectionally sequenced. No point mutations, deletions, insertions or polymorphisms were detected in the *NR3C1* gene of the 22 subjects.

3.3 | Real-time PCR analysis demonstrated no differences in LTL between glucocorticoid-resistant and glucocorticoid-sensitive subjects

To investigate the relationship between LTL and tissue sensitivity to glucocorticoids, we measured the average telomere length using multiplex monochrome quantitative real-time PCR. We found no difference in LTL between the glucocorticoid-resistant and the glucocorticoid-sensitive groups (Figure 1).

3.4 | RNA sequencing analysis revealed differential expression of genes between glucocorticoid-resistant and glucocorticoid-sensitive subjects

Cluster analysis of RNA sequencing revealed variability between glucocorticoid-resistant and glucocorticoid-sensitive subjects (Figure 2A). One hundred thirty-three genes were upregulated and 49 were downregulated in the glucocorticoid-resistant

TABLE 1 Endocrinological parameters in the glucocorticoid-sensitive and glucocorticoid-resistant groups

	Glucocorticoid-sensitive (n = 11)	Glucocorticoid-resistant (n = 11)
ACTH (pg/mL)	33.16 ± 5.67	27.64 ± 4.65
CORT (μg/dL)	23.13 ± 1.70	18.98 ± 3.06
IGF-I (ng/mL)	259.18 ± 23.97	251.36 ± 20.15
IGFBP-3 (μg/mL)	5.30 ± 0.31	5.17 ± 0.37
TSH (μUI/mL)	2.79 ± 0.28	2.05 ± 0.33
T3 (ng/dL)	102.26 ± 8.33	102.02 ± 7.19
FT4 (ng/dL)	1.12 ± 0.04	1.06 ± 0.03
Anti-TPO (IU/mL)	10.43 ± 0.21	11.11 ± 0.78
Anti-TG (IU/mL)	20 ± 0.00	20 ± 0.00
LH (mUI/mL)	10.11 ± 4.50	6.44 ± 0.69
FSH (mUI/mL)	5.22 ± 0.83	4.05 ± 0.70
E2 (pg/mL)	102.99 ± 32.08	69.05 ± 22.74
Testosterone (ng/dL)	187.77 ± 69.83	293.27 ± 84.51
DHEAS (μg/dL)	238.62 ± 44.03	248.58 ± 34.68
Androstenedione (ng/mL)	2.89 ± 0.28	3.20 ± 0.35
PRL (ng/mL)	24.94 ± 2.65	21.55 ± 2.75
SHBG (nmol/L)	65.12 ± 8.42	46.17 ± 5.09
PTH (pg/mL)	34.15 ± 4.59	38.51 ± 5.40
25-Hydroxyvitamin D (ng/mL)	16.06 ± 2.38	14.02 ± 2.56

Note: Data are presented as mean ± standard error of the mean (SEM).
Abbreviations: ACTH, adrenocorticotropic hormone; Anti-Tg, thyroglobulin antibodies; Anti-TPO, thyroid peroxidase antibodies; CORT, cortisol; DHEAS, dehydroepiandrosterone sulphate; E2, oestradiol; FSH, follicle-stimulating hormone; FT4, free thyroxine; IGF1, insulin-like growth factor 1; IGF-BP3, insulin-like growth factor-binding protein 3; INS, insulin; LH, luteinizing hormone; PRL, prolactin; PTH, parathormone; SHBG, sex hormone-binding globulin; T3, triiodothyronine; TSH, thyroid-stimulating hormone.
$P > 0.05$ for all.

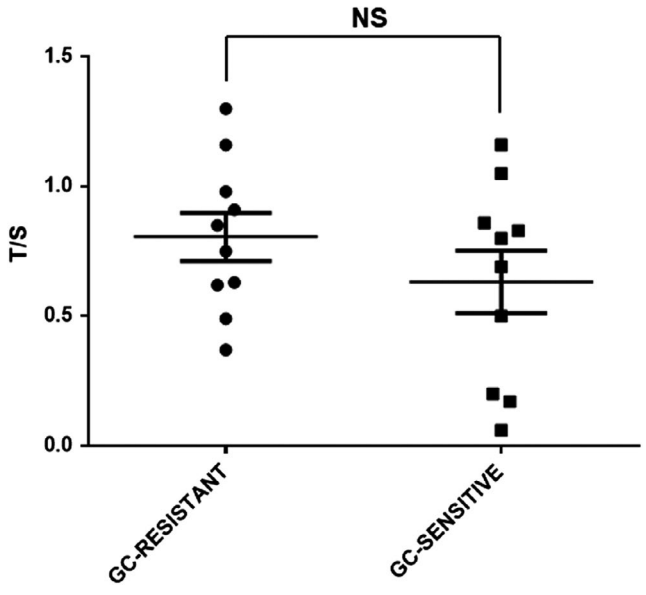

FIGURE 1 Mean leukocyte telomere length (T/S ratio) in glucocorticoid-resistant and glucocorticoid-sensitive subjects. NS, nonsignificant statistical difference; T/S, telomere-to-b-globin gene

compared to the glucocorticoid-sensitive group (Figure 2B). The majority of these differentially expressed genes were implicated in inflammatory response, immune response and NF-κB signalling pathway (Figure 3A,3). Further cluster analysis in the expression profile of the 22 subjects revealed differences between 3 glucocorticoid-sensitive and 3 glucocorticoid-resistant subjects. We found 1058 upregulated genes and 1139 downregulated genes in the 3 glucocorticoid-resistant compared to the 3 glucocorticoid-sensitive subjects (Figure 4). A large percentage of these differentially expressed genes play an important role in telomere maintenance (Figure 5A), systemic lupus erythematosus (Figure 5B) and Alzheimer's disease (Figure 5C). Interestingly, Synuclein A, a key molecule in Parkinson's disease, was found upregulated in the subgroup of glucocorticoid-sensitive subjects (Figure 5D).

4 | DISCUSSION

In this study, we showed that healthy subjects with differences in tissue sensitivity to glucocorticoids have distinct

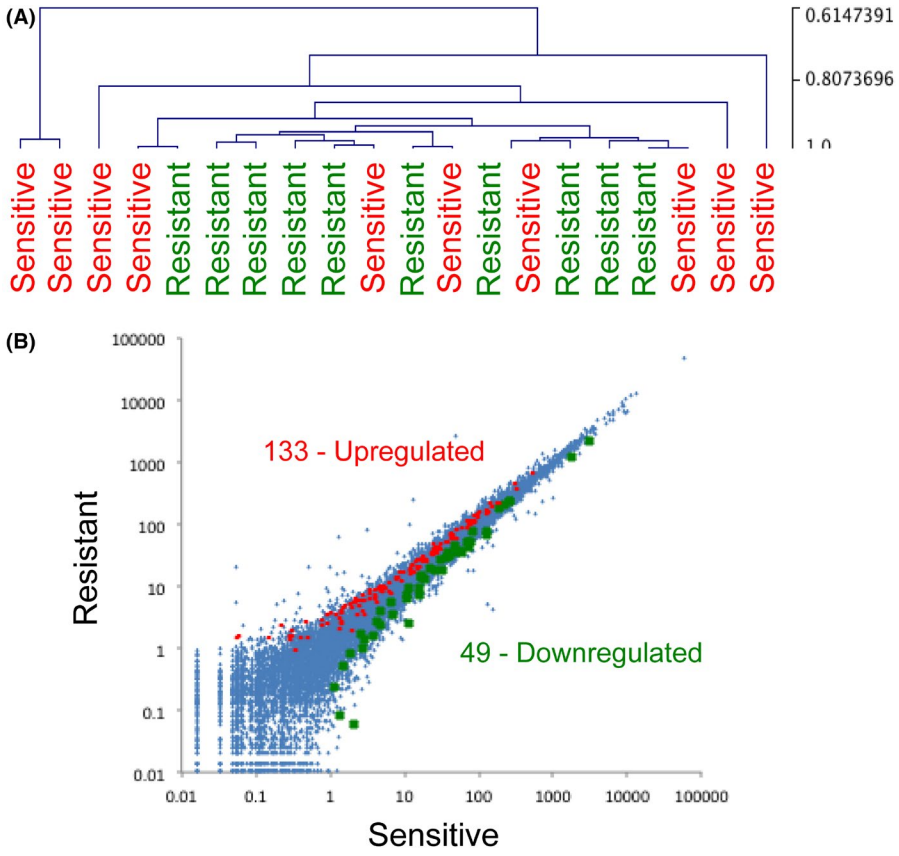

FIGURE 2 A, Transcriptomic analysis revealed variability between glucocorticoid-resistant and glucocorticoid-sensitive subjects. B, Differentially expressed genes in the glucocorticoid-resistant compared to the glucocorticoid-sensitive group

transcriptomic profiles. Importantly, none of the 22 subjects participating in the two groups harboured point mutations, deletions, insertions or polymorphisms in the *NR3C1* gene, indicating that the above-described variation in tissue sensitivity to glucocorticoids could not be attributed to genetic defects in the glucocorticoid receptor. Although there was a high variability between the two groups, transcriptomic analysis revealed 133 upregulated and 49 downregulated genes in the glucocorticoid-resistant compared to the glucocorticoid-sensitive group. Many of these differentially expressed genes were found to play a role in inflammatory/immune response and NF-κB signalling pathway. Among the 22 subjects of both groups, we further studied the differences of 3 glucocorticoid-sensitive and 3 glucocorticoid-resistant subjects at the transcriptomic level. We found 1058 upregulated genes and 1139 downregulated genes in the glucocorticoid-resistant compared to the glucocorticoid-sensitive subgroup. The majority of these genes implicated in telomere maintenance, systemic lupus erythematosus and neurological disorders, such as Alzheimer's and Parkinson's disease. Synuclein A, an important protein involved in the pathogenesis of Parkinson's disease, had increased expression levels in the glucocorticoid-sensitive subgroup.

It is important to note that in our study we did not find any genetic defects in the *NR3C1* gene in the 22 participants, which indicates that the differences at the transcriptomic level between the glucocorticoid-resistant and the glucocorticoid-sensitive groups occur independently of hGR expression and/or transcriptional activity. These results are consistent with and indirectly support the findings by Maranville *et al* who showed that glucocorticoid sensitivity was influenced by gene variants located in the cis-regulatory elements of glucocorticoid-responsive genes, rather than by genetic variants in master regulators, such as the *NR3C1* coding region and intron-exon junctions, several interacting transcription factors.[29] We speculate that likely cis-regulatory polymorphisms, which were not investigated in this study, might have an impact on glucocorticoid sensitivity.

To investigate whether tissue sensitivity to glucocorticoids influences LTL, we measured the average telomere length in the leukocytes of the 22 subjects and found no differences between the two groups. However, transcriptomic analysis showed that the glucocorticoid-sensitive group had increased expression of the majority of genes involved in telomere maintenance. We speculate that a number of glucocorticoid-sensitive subjects might have induction of gene expression implicated in telomere length as a counter-regulatory mechanism against acute or chronic stressors, granted that any excessive or prolonged activation of the stress system causes shortening of the telomere length.[30,31]

In addition to telomere maintenance, the glucocorticoid-sensitive group had increased expression of genes implicated

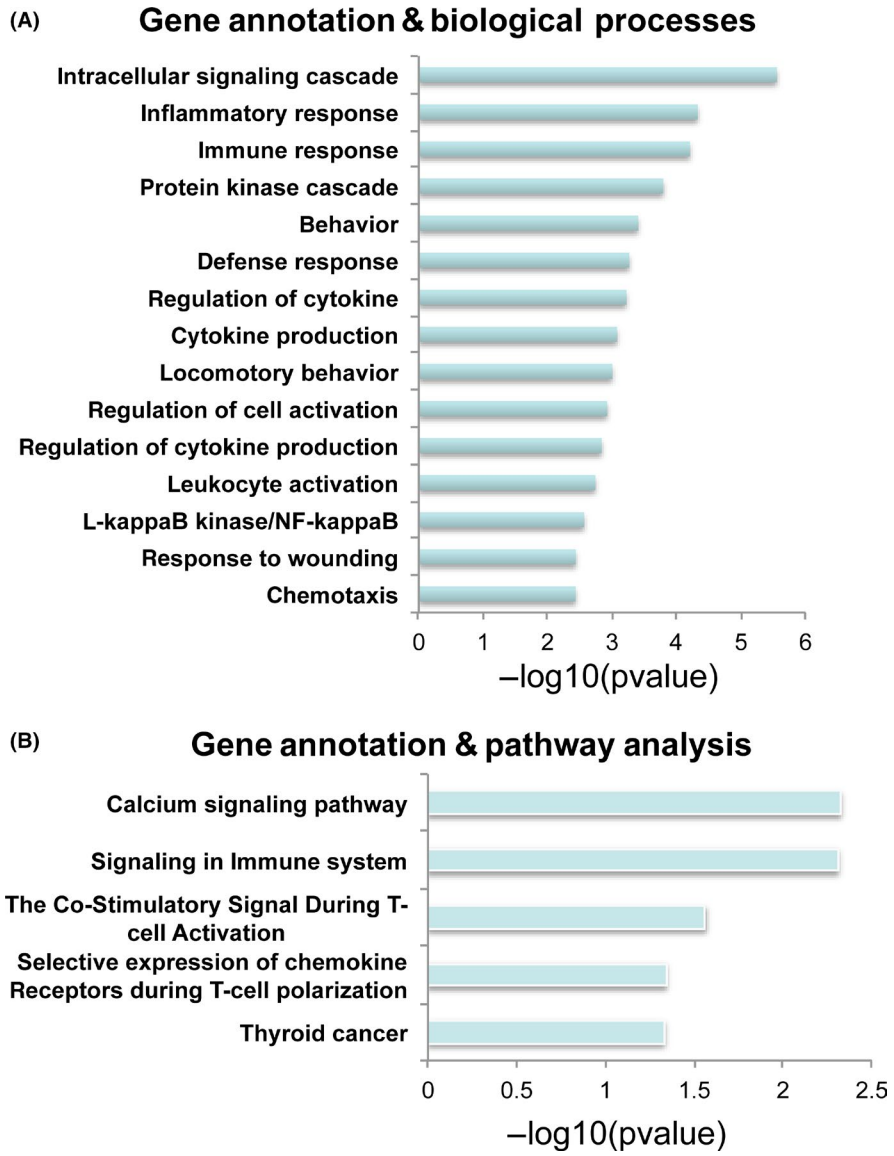

FIGURE 3 A, Top significant biological processes and (B) pathways. Hypergeometric test was used to determine the significant of a process or pathway ($P < 0.050$)

in the pathogenesis of systemic lupus erythematosus. This autoimmune disorder has been associated with a glucocorticoid-induced switch from Th1 to Th2 response[32]; therefore, healthy subjects with increased sensitivity to glucocorticoids might be more susceptible to develop systemic lupus erythematosus. Indeed, accumulating evidence suggests that a more potent response to stressors and immune/inflammatory signals may account for many contemporary stress-related and autoimmune disorders, including depression, anxiety, eating disorders, as well as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis.[33] Interestingly, a recent study by Duma *et al* demonstrated an augmented stress and immune/inflammatory response in the female gender, which might explain the higher incidence of the above-mentioned disorders in women than in men.[34] However, since the molecular pathogenesis of systemic lupus erythematosus is multifactorial, we hypothesize that increased glucocorticoid sensitivity might be one of the many components that synergistically lead to the clinical manifestations of systemic lupus erythematosus.

Finally, transcriptomic analysis showed differences between glucocorticoid-resistant and glucocorticoid-sensitive healthy subjects, which are associated with differential expression of genes related to neurological disorders. Interestingly, subjects in the glucocorticoid-resistant subgroup have increased expression of genes involved in Alzheimer's disease. This is in line with previous studies showing a decreased sensitivity of peripheral tissues to glucocorticoids in patients with Alzheimer's disease.[35] On the other hand, we found that there was increased expression of synuclein A in the glucocorticoid-sensitive compared to the glucocorticoid-resistant subgroup. This finding is in accordance with a recent study, which showed that

dexamethasone increased the expression of two genes implicated in Parkinson's disease, the LRRK2 and α-synuclein.[36]

Our study has some limitations. Our sample of 11 glucocorticoid-sensitive and 11 glucocorticoid-resistant subjects is small. Further studies with larger samples will undoubtedly shed light on the differences between glucocorticoid-sensitive and glucocorticoid-resistant subjects at the transcriptomic level. Moreover, it is not clear whether the differentially expressed genes affect glucocorticoid sensitivity, result from altered glucocorticoid action or are coincidental. Although some of these genes, such as α-synuclein, have been shown to be affected directly by glucocorticoids, we still do not know whether the rest of these genes influence glucocorticoid sensitivity or are differentially expressed due to an indirect effect of glucocorticoids. Furthermore, no functional analysis was performed in order to evaluate the effect of the DEGs on hGR.

In conclusion, transcriptomic analysis revealed differences in tissue sensitivity to glucocorticoids among healthy subjects. These differences are associated with the presence of differentially expressed genes related to autoimmune and neurological disorders.

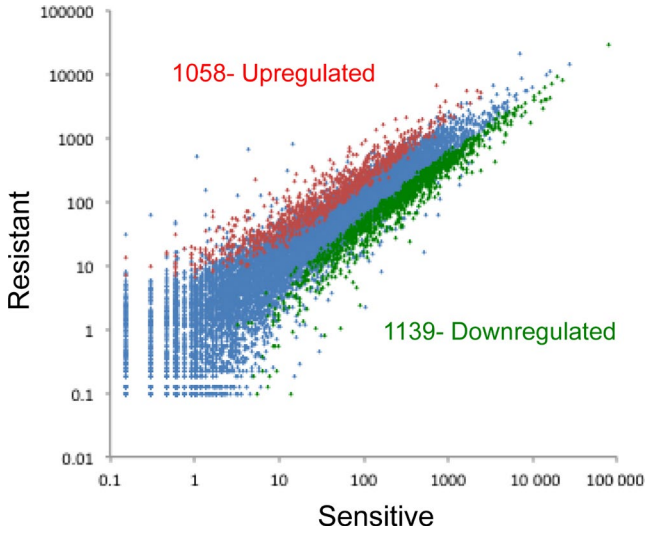

FIGURE 4 Differentially expressed genes in the glucocorticoid-resistant compared to the glucocorticoid-sensitive subgroup

CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

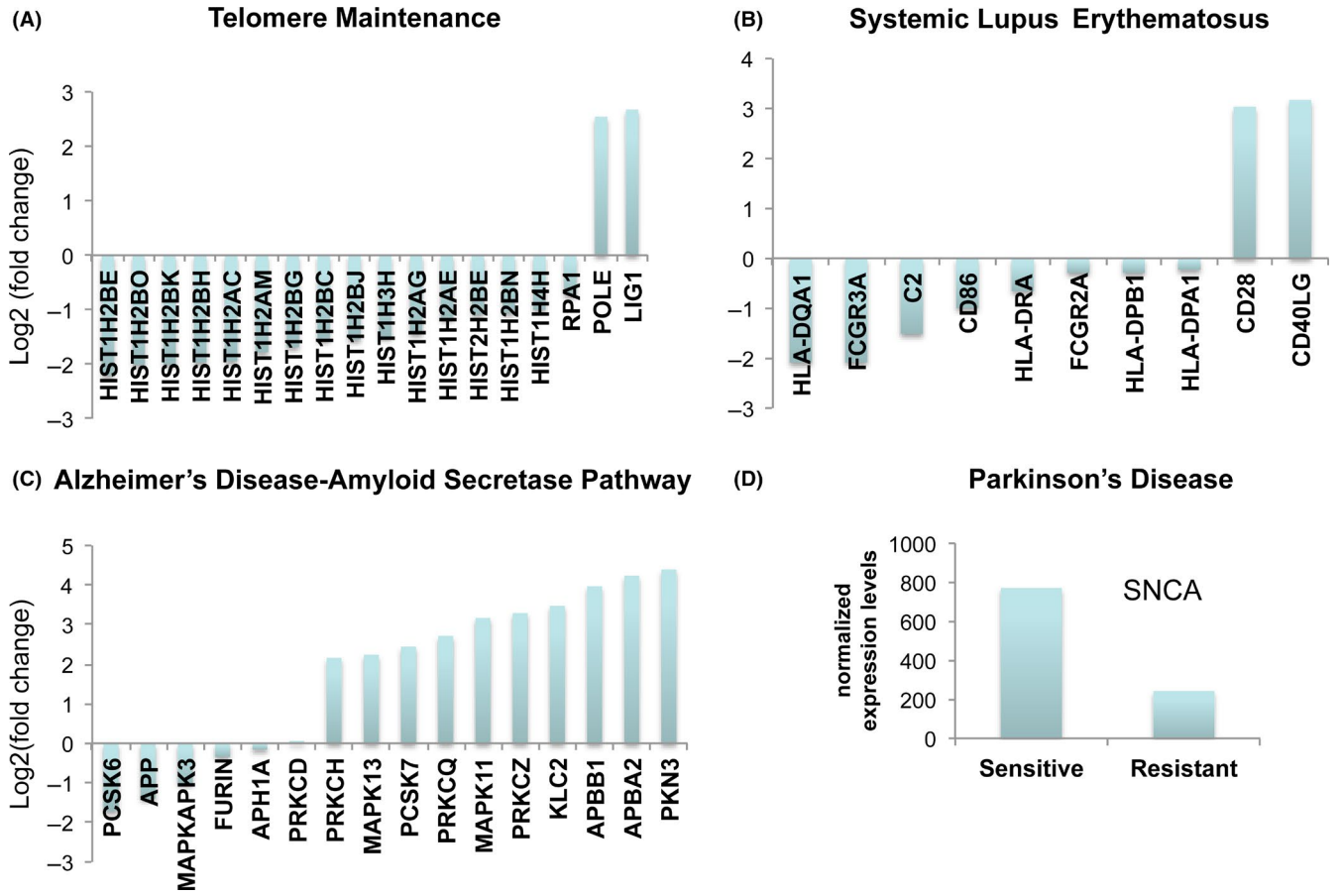

FIGURE 5 The majority of these differentially expressed genes were involved in (A) telomere maintenance, (B) systemic lupus erythematosus and (C) Alzheimer's disease. D, Synuclein A was found upregulated in the subgroup of glucocorticoid-sensitive subjects

AUTHOR CONTRIBUTIONS

Designed the study: EC. Designed the experiments: NCN, AP and AL. Performed the experiments: NCN, AP, AL and AS. Collected the data: NCN, EK, EG and CP. Analysed the data: NCN, AP, AL, IP, DT, GPC and EC. Wrote the paper: NCN, AP and EC.

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How to cite this article: Nicolaides NC, Polyzos A, Koniari E, et al. Transcriptomics in tissue glucocorticoid sensitivity. *Eur J Clin Invest*. 2019;e13129. https://doi.org/10.1111/eci.13129