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Glucocorticoid Receptors and Disease Activity in Systemic Lupus Erythematosus Patients

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Abstract: Glucocorticoids (GC) have been widely used to treat patients with systemic lupus erythematosus (SLE). However, GC-insensitivity remains a major barrier in achieving remission. Therefore, understanding its mechanism is crucial to enhance the efficacy of GC treatment. Our study aimed to explore the association of the nuclear receptor subfamily 3 group C member 1 (*NR3C1)* gene, which encodes GC receptors GRα and GRβ, the histone deacetylase2 (*HDAC2*), the histone acetyltransferase-1 (*HAT1*), and the interleukin- 23 receptor (*IL-23R),* with the response to treatment in SLE patients. **Methods:** Quantitative real-time PCR (qPCR) was used to determine the expression levels of *NR3C1* isoforms, *HDAC2*, *HAT1*, and *IL23R* in peripheral blood mononuclear cells (PBMC) from 30 SLE patients and 6 healthy controls (HC). Immunohistochemical (IHC) staining was used to analyze the protein expression level of GRα in 19 SLE patients' 8 controls renal biopsies. **Results:** GRα mRNA expression level was associated with SLE disease (p=0.018) and correlated with the SLEDAI score (p=0,038). HDAC2 was up-regulated in patients during remission phase compared to active phase (p=0.015). The IL23R mRNA expression was associated with anemia (p=0.037) and lymphopenia (p=0.028). It was up-regulated in treated patients with the combination of hydroxychloroquine, cyclophosphamide, and methylprednisolone (p=0,028) compared to other treatments. Tubular GRα expression showed a positive correlation with the chronicity index (rs=0.607, p=0.016). **Conclusion:** The GRα may be involved in the pathogenesis of SLE. It appears that HDAC2 contributes to the remission phase in SLE. IL23R expression could be affected by treatments in SLE patients.

Key words: Lupus, Glucocorticoids, Glucocorticoid receptors, HDAC2, IL23R.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex and severe auto-immune disease (AID) characterized by a variety of auto-antibodies (-Abs) and immune complexes directed to the target tissue and a persistent activity of pathogenic B and T cells ***(Oelke et Richardson 2004).*** SLE has varied clinical manifestations and involves multiple systems and organs. Glucocorticoids (GC) as inflammation inhibitors are the most effective drugs in the treatment of SLE. The majority of GC activity is mediated by GC receptors (GR). The human GR gene, also known as Nuclear Receptor subfamily 3 group C member 1 (*NR3C1*), is located on chromosome 5q31-32 and is composed of 9 exons. It encodes several isoforms of GR protein. GRα and GRβ are the most important isoforms resulting from the alternative splicing of the primary GR transcript. GRα can bind to the GC and then translocate to the nucleus for regulating gene expression ***(Ito, Chung, et Adcock 2006)***. However, GRβ is constitutively expressed in the nucleus but cannot bind to the ligand or mediate anti-inflammatory GC effects ***(Bamberger et al. 1995), (Bledsoe et al. 2002)***. Therefore, GRβ acts as a negative dominant over GCs despite the absence of direct binding to GC. Three mechanisms have been hypothesized to explain GR's negative activity ***(Bamberger et al. 1995), (Bledsoe et al. 2002)***: (i) GRβ competes with GRα for binding to DNA's GC response elements (GRE), (ii) GRβ generates non-transactivating heterodimers by binding to GRα and (iii) GRβ reduces the quantity of GRα coactivators ***(Bamberger et al. 1995), (Bledsoe et al. 2002)***. On the other hand, GCs exert their anti-inflammatory effects by influencing multiple signal transduction pathways including the ability to induce apoptosis in T and B lymphocytes ***(Tuckermann et al. 2005).*** Their most important action is switching off multiple activated inflammatory genes through the inhibition of histone acetyl-transferases (HAT) and recruitment of histone deacetylases (HDAC) activity to the inflammatory gene transcriptional complex. Indeed, HAT and HDAC are families of enzymes which regulate chromatin structure, and through this, affect inflammatory genes' expression ***(Wolffe et Matzke 1999)***. There are two main types of HAT: type A and type B. Type A HAT are typically found in the nucleus and add acetyl groups to histones on chromatin, while type B HAT are found in the cytoplasm and primarily acetylate newly synthesized histones before they are incorporated into chromatin ***(Marmorstein 2001)***. Specifically, HAT1 is an enzyme that belongs to the type B HAT family which is involved in acetylating newly synthesized histone H4 during DNA replication. This acetylation is important for proper chromatin assembly and regulation of gene expression ***(Tropberger et al. 2013)***. It has been shown that HAT1 can moderate hyperinflammation by decreasing NF-κB signaling, the IFN-I response, and IL-6 production ***(Du et al. 2014), (Sadler et al. 2015)***. However, HDAC2 may play an important role in deacetylating the acetylated GR, including the estrogen and androgen receptors ***(Fu et al. 2004), (Barnes 2006), (Ito et al. 2006)***, after GC binding so that it can repress NF-kB-regulated inflammatory genes.

In addition to the well-known molecules implicated in the response to GC, the interleukin 23 receptor (IL-23R) was also proposed to play a role in response to several treatments including GC. Il23R serves as an initial sensor of IL-23 for the Th17 cell-mediated autoimmune responses ***(Abraham et Cho 2009), (Neurath 2007)***. Indeed, the *IL23R* gene that is mapped to chromosome 1p32.1-p31.2, has been identified as a susceptibility gene to inflammatory and AID by genome-wide association studies ***(Duerr et al. 2006), (Kim et al. 2011), (Abraham et Cho 2009), (Neurath 2007), (Vermeire, Van Assche, et Rutgeerts 2010), (Sarra et al. 2010)***. So far, *IL-23R* polymorphisms were found to be associated with a lower chance of responding to GC in inflammatory bowel disease patients ***(Kim et al. 2011), (Cravo et al. 2014).***

The aim of this study is to identify biomarkers that can predict GC-insensitivity in SLE patients. To this end, we evaluated the mRNA expression levels of NR3C1 isoforms, HAT1, HDAC2, and IL23R in SLE patients compared to healthy controls, and their relationship with their immunological and clinical characteristics. Additionally, we sought to evaluate the tissue expression of GRα in renal biopsies.

METHODS

Patients and controls

This study was approved by the ethics committee of the University Hospital Habib Bourguiba of Sfax, Tunisia (protocol number of the ethics committee, 02/14). Before enrolment into the study, we obtained written informed consent from all participants.

We performed a retrospective study, enrolling 30 SLE patients attending the Internal Medicine department of the University Hospital Hedi Chaker of Sfax, Tunisia and who fulfilled the 1997 Update of the 1982 American College of Rheumatology (ACR) Revised Criteria for Classification of SLE ***(Hochberg 1997)***. Patients were recruited based on their response to GC therapy following the classification of ***(Lou et al. 2021)***. Thus, the SLE Disease Activity Index (SLEDAI) was taken during the first discovery of the disease $(SLEDAI)_0$ and after 12 weeks of the discovery of the disease $(SLEDAI)_{12}$. Indeed, GC-sensitive group is defined as patients whose $(SLEDAI)_{12}$ score was ≤ 4 and patients whose $(SLEDAI)_{12}$ was >4 but improved by ≥ 5 after treatment, and GC-insensitive group defined as patients who did not meet the above criteria, or, due to the lack of efficacy during treatment, used other immunosuppressive agents. An exhaustive information sheet containing clinical, serological, immunological, and histological features as well as therapeutic plans and SLEDAI score was filled out for each patient in two different times of the disease (at the first discovery of the disease and at the sample collection day (7.43±1.23 years of the disease duration).

Drug-induced lupus and pregnant patients were excluded from the study.

Patients included in this study received GC, immunosuppressive and antimalarial treatments (Prednisolone, Methylprednisolone, Cyclophosphamide, Hydroxychloroquine) as monotherapy or in combination with other treatments at various doses. The type of therapy was based on the patient's disease state.

To improve the association analysis between the expression level of the studied molecules and lupus, SLE patients were divided into different groups (supplementary data: figure S1) based on:

- SLEDAI scores at the sampling time:

(1) No activity (SLEDAI=0; n=12), mild (SLEDAI=1 to 5; n=7), moderate (SLEDAI=6 to 10; n=7) , high and very high (SLEDAI=11 to 19; SLEDAI≥20 ; n=4) ***(Griffiths, Mosca, et Gordon 2005)***.

(2) Active Lupus (SLEDAI <6 ; n=12). vs. inactive Lupus (SLEDAI≥6 ; n=18)

- Response to GC treatment after 12 weeks of the first discovery of the disease:

(1) GC-sensitive (n= 22) or GC-insensitive (n=8) groups defined by Zou *et al* (Zou et al. 2013) as*:* patients with clinical remission of symptoms, and a $(SLEDAI)_{12} < 5$, versus patients who showed no remission, and had $(SLEDAI)_{12} \geq 5$ or those who required additional immunosuppressive agents.

- State of the disease at the sampling time:

(1) Clinical remission phase (n=21) vs. active chronic phase (n=9).

In this study, 25 SLE patients (83%) were diagnosed with lupus nephritis (LN), 15 of them were included in our immunohistochemical study. Six healthy control (HC) volunteers originating from the South of Tunisia and aged between 25 to 50 years old, who did not suffer from any autoimmune or inflammatory disease, were included.

Sampling

(i) Blood: Ten ml peripheral blood samples were collected in Ethylenediamine tetraacetic acid (EDTA) tubes from SLE patients and 6 HC for mRNA expression analysis.

(ii) Biopsies: Eighteen embedded paraffin renal biopsies of LN patients were collected from the Pathology department of the University Hospital Habib Bourguiba of Sfax, Tunisia. Patients were followed up in the Nephrology department of the University Hospital Hedi Chaker of Sfax, Tunisia (age, sex, and duration of the disease are mentioned in Table 1) and classified according to the International

Society of Nephrology/Renal Pathology Society classification of LN (2003ISN/RPS) ***(Weening et al. 2004)*** as follows: 1 with class II, 1 with class II+V, 2 with class III, 3 with class IV, 3 with class V, 5 with class IV+V, 1 with II+V and 2 III+V. The immunofluorescence tests data for IgG, IgM, IgA, C1q, C3, kappa light chain, lambda light chain, and fibrinogen deposits were collected for each biopsy. The activity and sclerosis index were used to assess disease activity and cumulative damage. Four renal tissues obtained from the normal part of nephrectomized kidneys (because of renal carcinoma) and 4 cadaver kidneys (autopsy) from subjects without renal disease served as normal controls. The absence of cellular infiltrate and inflammation has been confirmed by histological examination by hemotoxylin and eosin staining.

Immune cells isolation

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using the Ficoll 1.077 (Eurobio®, France) density-gradient method. Viability and purity of PBMC were checked and evaluated by trypan in light microscopy. The cell suspension was lysed with Trizol reagent (Invitrogen®, Massachusetts, USA) and stored in Eppendorf tubes at -80°C.

Total RNA isolation and real time -PCR assay

Total RNA was extracted from the Trizol suspension according to the manufacturer's instructions. The RNA purity and integrity in each sample were assessed using a NanoDrop system (NanoDrop Technologies®, NC, USA) and using standard agarose gel electrophoresis. RNA was reversely transcribed using the PrimeScript RT Reagent Kit (TAKARA Bio®, Japan). Quantitative real-time PCR (qPCR) was performed with Real-Time PCR Detection System and TB Green™ Premix Ex Taq™ (TAKARA®, Japan) to quantify the *NR3C1* variant *GRα* (GenBank access number X03225), *GRβ* (X03348.1), *IL-23R* (NM_144701), *HAT1* (NM_003642) and *HDAC2* (NM_001527) transcripts. The transcripts of human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, NM_001357943.2) served as house keeping gene (Table S1). All reactions were performed in duplicates. For verification of the quality of PCR products, melting curves and electrophoresis migration were generated. The relative quantification was performed using the standard curve method and expression levels were estimated by the expression method ($2^{-\Delta Ct}$).

Immunohistochemical analysis

Immunohistochemical (IHC) staining was performed using the primary Ab for GRα. Paraffin-embedded tissue sections, 3–4 μm thick, were mounted on positively charged slides and heated at 60°C for 30 min, deparaffinized and rehydrated through a series of alcohols before staining. After antigen retrieval using 10 mM sodium citrate (pH=6) buffer microwaved for 15 min, endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min. Sections were washed three times with cold 0.01 M phosphate-buffered saline (PBS) after blocking with 0.4% buffered casein solution. Slides were then incubated overnight at 4°C with anti-GRα Ab (ab3580; Abcam®, UK) at 1: 200 dilutions. After 20 min incubation with the secondary Ab [a poly-horseradish peroxidase (HRP)-IgG-anti-rabbit designed by Novolink Polymer (Leica Biosystems®, UK), a prepared diaminobenzidine (DAB) substrate chromogenic solution was applied and incubated for approximately 10 min until color intensity was reached. Finally, sections were counterstained lightly with hematoxylin and examined under light microscopy (Axiolab, Zeiss®, Germany) and were fitted with a Power Shot camera (A 640, Canon®, Japan) to capture images for histological studies. The interpretation was carried out jointly by a pathologist and a nephrologist. For negative control preparation, the primary Ab was replaced with irrelevant isotype-matched control immunoglobulin (data not shown).

Biopsy IHC scoring

For each section, GRα expression score was assigned by giving a score ranging from 0 to 12 resulting in the product: intensity score × diffusion score based on the strategy adopted in the studies of Elloumi *et al.****(Nesrine Elloumi et al. 2017)*** **and** ***(N Elloumi et al. 2017)***. The intensity score ranged from 0 to 3: 0 for negative, 1 for weakly positive, 2 for moderately positive and 3 for strongly positive staining. The diffusion score ranged from 0 to 4 depending on the ratio of the stained tubules or glomerular to the

total observed tubules or glomerular of each specimen: 0 for 0%, 1 for 0–25%, 2 for 25–50%, 3 for 50–75% and 4 for > 75%.

Statistical analysis

All statistical analysis was performed using the SPSS software package (26.0 SPSS Inc., IL, USA). Continuous variables were expressed as mean± SEM. Non-parametrical multiple comparison tests, The Mann-Whitney U and Kruskal–Wallis tests, were applied for statistical analyses between the experimental groups. GraphPad Prism 5.0 was used to plot the graphs presented as the mean ± SEM. The spearman's rank-order test was used to analyze the correlation between the mRNA and protein expression pattern and the quantitative clinical and serological data. For all analyses, the value of $p < 0.05$ was considered statistically significant.

RESULTS

Epidemiological and clinical characteristics of the studied SLE patients

A total of 30 SLE patients were enrolled in our retrospective study. The Clinico-epidemiological and immunological characteristics are detailed in table 1. The average age of the disease onset and the disease duration in patients were 31.93±2.47 and 7.43±1.23; respectively. At the sampling time, the most common clinical feature was LN (83%). Lymphopenia was present in 26% of cases followed by anemia and photosensibility (23%) and malar rash (MR) (13%). The absence of arthritis in our SLE patients was noteworthy.

The anti-nuclear Abs (ANA) were present in all patients. Complement 3 (C3) and complement 4 (C4) serum levels were low in 65% of cases. The anti-dsDNA Ab and the anti-SSA Ab were present in 62% of cases followed by anti-RNP and anti-Ro-52 Abs found in 55% of cases. The absence of anti-Ribosome Abs (anti-Rib-P) was notable.

The clinical and immunological features of SLE patients at the first diagnosis are mentioned in supplementary table S2.

The corticosteroids and immunosuppressive treatments details are mentioned in table 1. In terms of medications, prednisolone was administered at a dosage of 0.1 mg/kg/day to 1mg/kg/day. In 16 cases (46%), the prednisolone dosage was gradually diminished, since the response to treatment was considered good according to the clinical conditions. Fourteen of the patients experienced an aggravation of the disease, and methylprednisolone and/or cyclophosphamide were thus initiated as adjuvant therapy. Hydroxychloroquine was administrated as a long-term treatment to prevent any relapse. At the sampling time, 83% of patients were under hydroxychloroquine, followed by prednisolone (76%), in monotherapy or in combination with other treatments. The less administrated treatments in our population were methylprednisolone and cyclophosphamide (16% and 30%, respectively). Only 22% of patients were under monotherapy either with prednisolone (16%) or hydroxychloroquine (6%). Almost 75% of patients were under conbination of treatments, most of them were under hydroxychloroquine and prednisolone together (30%).

Expression levels of GRα and GRβ genes

Baseline expression levels of GR isoforms' mRNAs were quantified in isolated PBMC from the 30 SLE patients compared to 6 HC. GRα mRNA expression was significantly higher in the HC group compared to the SLE patients' group (0.11±0.07 vs 0.024±002; p=0.018) (figure 1.a). However, no difference in GRβ mRNA expression was found between the two groups (0.000036±0.000008 vs 0.00021±0.00017; p=0.63). The GRα / GRβ ratio was significantly up-regulated within female group (2162.16±482.4 vs 367.05±198.94; p=0.049).

On the other hand, the GRα mRNA expression level was up-regulated in patients without MR clinical feature compared to those with MR (0.028± 0.003 vs 0.01±0.003; p=0,033) (figure 1.b). GRβ expression level was not associated with any clinical feature. Regarding immunological features, no association was found with the GRα and GRβ expression levels.

Furthermore, no significant difference was observed in GRα and GRβ transcript levels among the different groups of patients classified according to the SLEDAI scores, the phase of the disease or the response to treatment.

Table 1: Demographical, clinical, immunological features and treatment plans of SLE patients

Demographical characteristics of SLE patients		
Patients	30	
M/F	3/27	
Age of the disease onset (year)	31.93±2.47	
Duration of disease (year)	7.43±1.23	
Clinical features of SLE patients		
Symptoms	**Patients (%)**	
Malar rash	13	
Photo sensibility	23	
Oral ulcers	3	
Anemia	23	
Arthritis	0	
Polyarthralgia	5	
Nephritis lupus	83	
Pleurisy	6	
Raynaud syndrome	3	
Pericarditis	6	
Lymphopenia	26	
Asthenia	10	
Fever	5	
Immunological features of SLE patients		
Auto-antibodies/components	**Positive patients (%)**	
ANA	100	
Anti-dsDNA Abs	62	
Anti-NUCLEOSOME Abs	44	
Anti-CENTROMERE Abs	3	
Anti-HISTONE Abs	41	
Anti-Sm Abs	37	
Anti-RNP Abs	55	
Anti-SSA Abs	62	
Anti-Ro-52 Abs	55	
Anti-SSB Abs	17	
Anti-Scl 70 Abs	0	
Anti-Ribosome Abs	0	
Anti-Mi 2 Abs	0	
Anti-DFS 70 Abs	6	
Complement 3 (C3)	65	
Complement 4 (C4)	65	
Rheumatoid factor	0	
Treatments for SLE patients		
Treatment	**Patients (%)**	**Doses (mg/d)** (mean±SEM)
Hydroxychloroquine	83	206.6±29.5
Prednisolone	76	13.9±3.2
Methylprednisolone	16	0.5–2 mg/kg/day in 2 successive weeks
Cyclophosphamide	30	-

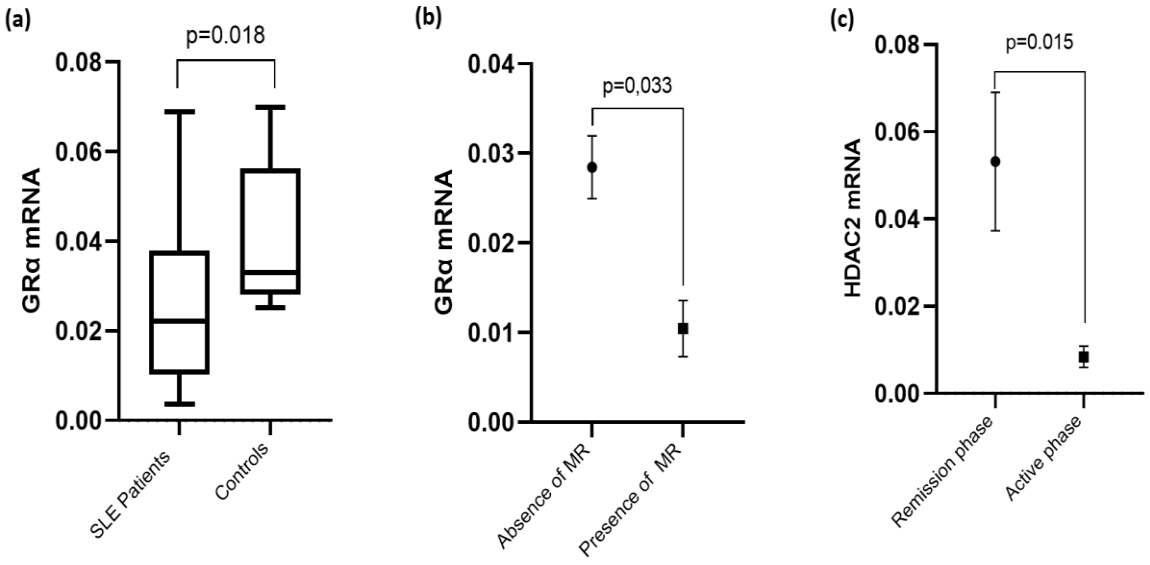

Figure 1: (a) GRα mRNA expression level up-regulated in HC (n=6) group compared to SLE patients (n=30). (b) GRα mRNA expression level was down-regulated in patients with presence of malar rash (MR) (n=4) compared to patients without malar rash (n=26). (c) HDAC2 transcript was up-regulated in patients in the remission phase (n=21) of the disease compared to patients in the active phase of the disease (n= 9).

Expression levels of HAT1 and HDAC2 genes

The mRNA expression levels of HDAC2 and HAT1 showed no significant association in SLE patients compared to HC (0.068±0.027 vs 0.01±0.03 and 0.93±0.9 vs 0.03±006, p=0.4 and p=0.2; respectively). No significant association was reported between HDAC2 and HAT1 transcript levels and sex, age, or any major clinical and/or immunological manifestation.

The classification of SLE patients according to the SLEDAI scores showed no significant difference in mRNA expression levels of HAT1 and HDAC2 among the different groups. However, the results revealed an up-regulation in HDAC2, but not in HAT1 transcript level in the remission phase of the disease compared to the active phase (0.053 ±0.015 vs 0.007± 0.02; p=0.015) (figure 1.c). No difference in HAT1 and HDAC2 mRNA expression was found between the two groups of GC-sensitive and GC-insensitive patients.

Expression level of IL23R gene

The mRNA expression level of IL23R showed no significant association in SLE patients compared to HC (0.0014±0.0007 vs 0.0017±0.001, p=0.7). Statistical data revealed a significant up-regulation of IL23R mRNA relative expression in SLE patients with lymphopenia compared to those without lymphopenia (0.0019±0.001 vs 0.0014±0.0007; p=0.028) (figure 2.a) and patients with anemia compared to those without anemia (0.0018±0.0009 vs 0.0002±0.00005; p=0.037) (figure 2.b). No association was found with any immunological feature.

The SLE clinical stratification of SLE patients according to the SLEDAI scores, the phase of the disease, or the response to GC treatment showed no difference in IL23R mRNA among the different groups.

Correlations between treatments and GRα, GRβ, HAT1, HDAC2 and IL23R expression levels

Regarding the association between different treatments and the gene expression levels, *IL23R* expression was up-regulated only in treated patients with a combination of hydroxychloroquine, cyclophosphamide, and methylprednisolone (p=0,028) compared to all other combination of treatments (figure 2.c). No association was found between GRα, GRβ, HAT1 and HDAC2 mRNA levels and any treatment.

The overall expression of all studied genes is recapitulated in supplementary figure S2.

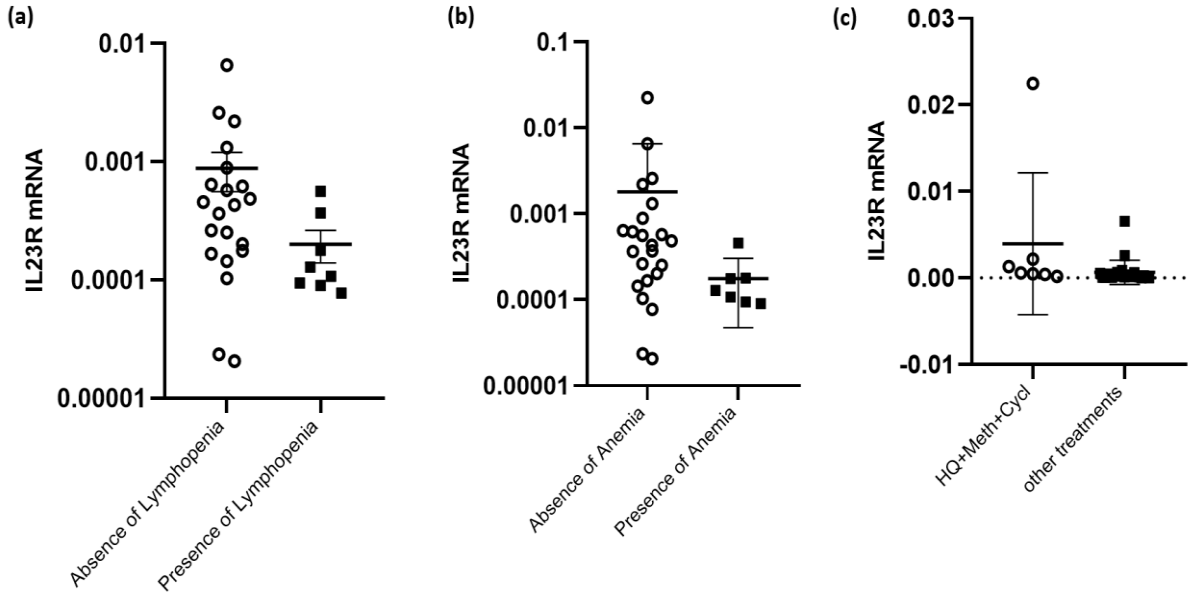

Figure 2: (a) IL23R mRNA expression level was down-regulated in the PBMC of patients with presence of lymphopenia (n=8) compared to patients that did not show lymphopenia (n=22) (p=0.028). (b) IL23R transcript was up-regulated in PBMC patients in absence of anemia (n=23) compared to patients that show the presence of anemia (n=7) (p=0.037). (c) IL23R mRNA expression level was down-regulated in SLE patients treated with Methylprednisolone + Hydroxychloroquine + cyclophosphamide (n=7) compared to patients treated with different other treatments (Hydroxychloroquine, methylprednisolone, cyclophosphamide and prednisolone) (n=23).

Expression of GRα protein in renal tissue

GRα was detected in all analyzed renal tissues in both controls and LN patients (figure 3). GRα-positive cells were observed in all renal compartments, and the antigen was mainly localized in tubular region. Statistical analysis revealed no difference in the protein expression levels between LN biopsies and controls neither in tubule nor in glomeruli (3,48±0,19 vs 3,29±0,66 and 1±,63 vs 1,11±0,14, respectively). Our results indicated an association between the distribution of tubular GRα protein expression and fibrinogen (p=0.014). Also, an association was observed between the intensity of tubular GRα protein expression and cellular debris (p=0.038). Additionally, tubular GRα expression showed a positive correlation with the chronicity index (p=0.016, rs=0.607). No association was found between glomerular GRα protein expression score and any immunohistological parameter except glomerular basement membrane abnormality (p=0.023). Indeed, the glomerular GRα protein expression was down-regulated in LN patients with glomerular basement membrane abnormality compared to LN patients with normal glomerular basement membrane (2,25±1,25 vs 0,2±0,2).

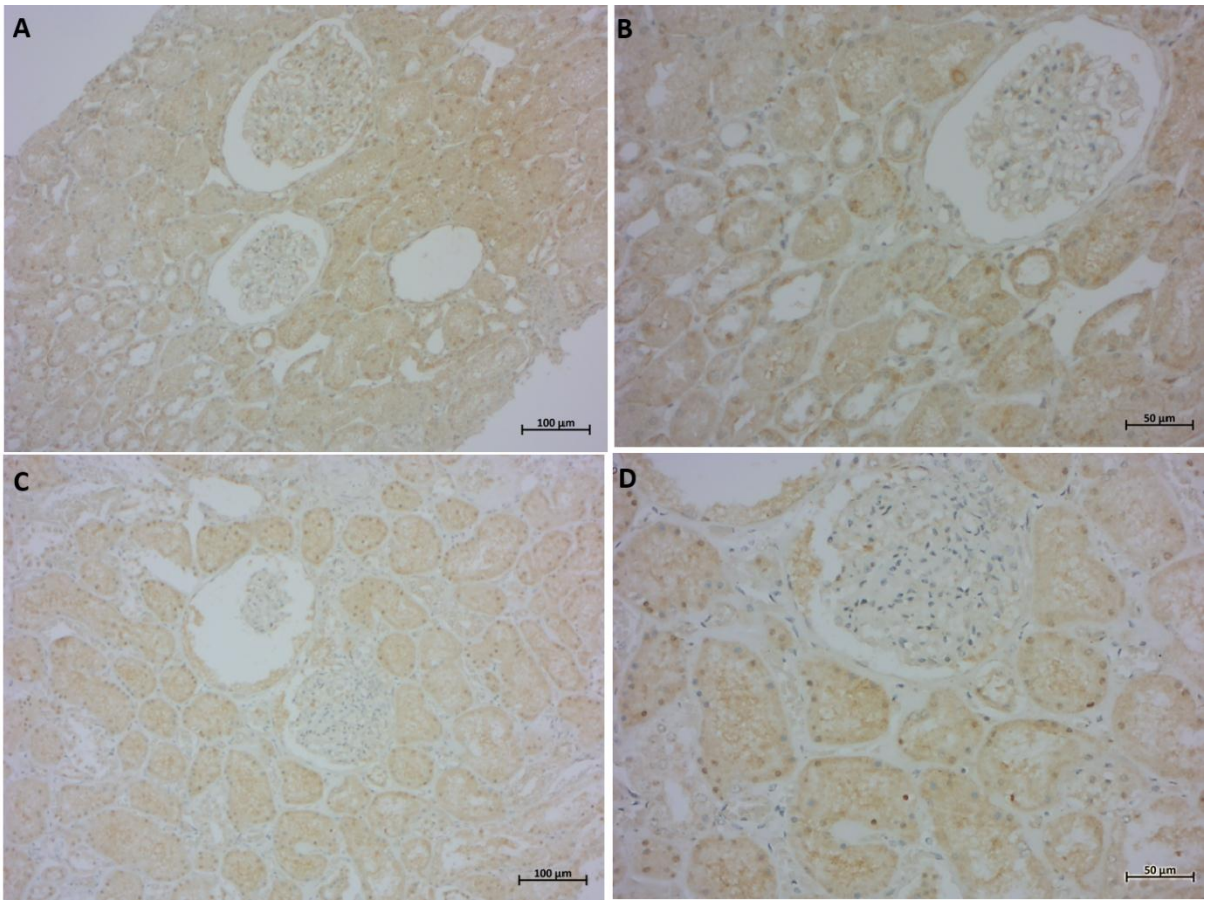

Figure 3: GRα expression in glomeruli and tubules in LN patient and control: (A) in LN patient (Magnification: ×100; scale bar:100µm) (B) in LN patient (Magnification: x200, scale bar; 50µm). (C) in control (Magnification: ×100; scale bar:100µm) (D) in control (Magnification: x200, scale bar; 50µm) Abbreviations: GRα, Glucocorticoid receptor α; LN, lupus nephritis. tubules and glomeruli show predominantly nuclear staining for GR α in patients compared to control without significant difference.

Discussion

Uncertainty exists regarding the underlying molecular mechanisms of GC therapy on SLE disease, especially the inflammatory mediators linked to GC insensitivity. In this study, we performed PBMC' gene expression profiling and proteomic analysis of the major receptors of GC (*GRα* and *GRβ*), epigenetic modification analysis through the antagonists (*HAT1*, *HDAC2*), and the key mediator of IL-23/IL-17 axis, *IL23R* in SLE patients. Our results showed a significantly low expression level of *GRα* in SLE patients compared to HC. A similar observation was reported in the Chinese population ***(Li et al. 2010), (Wang et al. 2012)***. Chen H. *et al.* suggested that the low expression of *GRα* gene in SLE patients is due to hypermethylation of *GRα* promoter ***(Chen et al. 2015)***. This lends credence to the hypothesis that reduced *GRα* expression levels play a significant role in the pathophysiology of SLE. In contrast to the suggested dominant-negative effects of GRβ on GRα-mediated responses, *GRβ* mRNA expression levels were equal in SLE patients and HC. The same result has been reported in Crohn's disease patients compared to HC ***(Hori et al. 2002)***. However, a previous study showed an up-regulation of *GRβ* mRNA level in SLE patients compared to HC in the Chinese population ***(Wang et al. 2012)***. There is evidence from ***(Hecht et al. 1997)*** indicating that GRβ does not serve as a significant repressor of GC action physiologically. ***(Oakley, Sar, et Cidlowski 1996)*** found that the amount of *GRβ* mRNA is only 0.2-0.3% of total GR mRNA, suggesting that it may not have any effect on GRα action.

Regarding the response to treatment, *GRα* level is correlated with the clinical response to GC treatment in a variety of diseases including rheumatoid arthritis ***(Schlaghecke et al. 1992)*** and LN

(Shahidi et al. 1999). In contrast, GRβ, which is thought to play an important role in the downregulation of GC action, is reported to be associated with resistance to GC treatment in ulcerative colitis ***(Honda et al. 2000)*** and asthma ***(Hamid et al. 1999)***. However, our results did not show any association between the GC treatment and the expression level of both isoforms. This may be due to the genetic background of the Tunisian population or perhaps to the limited size of our sample. Indeed, has shown a significant association between GR genetic polymorphisms and the efficacy of GCs in patients with SLE which could increase GRβ protein expression and decrease GRα protein expression in the Polish population ***(Zou et al. 2013).***

The study revealed a negative correlation between SLEDAI score and *GRα* expression level which has been reported by ***(Wang et al. 2012)*** and ***(Guan et al. 2015)***. These findings strongly agree with previous studies which found that both GRα mRNA and protein expressions were down-regulated in the SLE steroid-sensitive patients group and negatively correlated with the SLEDAI score ***(Zou et al. 2013), (Li et al. 2010)***. In addition, our results showed a down-regulation in *GRα* mRNA expression level in patients with MR compared to those without MR. It seems that *GRα* expression prevents skin damage. On the other hand, the evaluation of the GRα protein levels in LN patient' biopsies compared to controls revealed no significant difference, which has been in SLE patients ***(Piotrowski et al. 2007)*** and contradicts the previous results reported by ***Tziraki et al. (2007)*** as the number of GR-positive podocytes in controls was significantly higher than in the treated and untreated LN patients in their Greek population**.**

These conflicting data, combined with our current findings, discuss the role of both GRα and GRβ in the clinical effectiveness of GC in SLE patients and may reflect inter-individual differences in SLE patients regarding their adaptation capacity in GR regulation.

HAT is associated with genes' activation, whereas *HDAC* activity is associated with the silencing of genes. Therefore, *HDAC* has been targeted as a potential therapeutic protein for several diseases, including SLE. Methylation of *HDAC2* as well as, changes in histone acetylation status are known to occur in lupus patients. Our results show no significant difference between *HDAC2* mRNA levels in SLE patients compared to HC. The same observation was reported in asthma ***(Cosío et al. 2004)***. In contrast, Hu *el al*., found that *HDAC2* transcript levels were considerably lower in SLE patients compared to HC in the Chinese population ***(Hu et al. 2008)***, While another study showed that *HDAC 2* expression was higher in SLE patients compared to African American and European American controls ***(Kenneth L et al. 2018)***. Moreover, our results revealed a significantly higher level of the *HDAC2* mRNA in the remission phase compared to the active phase of the SLE disease. This expression was negatively correlated with the disease activity. Our result corroborates previous studies which have shown that *HDAC2* level is downregulated in active SLE $CD4^+$ T cells ***(Hu et al. 2008)***, but contradicts others which reported an increased expression of *HDAC2* in SLE patients with SLEDAI ≥4 compared to those with SLEDAI<4 ***(Kenneth L et al. 2018)***. Tacking together, these results confirm the importance of epigenetic alterations (histone acetylation) in SLE etiology and suggest that the HDAC2 increased expression could be an indication of SLE disease activity.

Studies focusing on the *IL23R* role have been arising in recent years. Indeed, *IL-23R* has been proposed as a common genetic marker for a variety of AID such as SLE ***(Leng et al. 2010).*** In the present study, we discuss the relationship of *IL23R* with the clinical/immunological features and treatment of SLE. No difference was found in the *IL23R* expression level in SLE patients compared to HC. The expression level was significantly higher in the presence of anemia and lymphopenia. Indeed, A study conducted earlier suggested that a functional variant of *IL23R* (rs11209032) may be linked to the occurrence, severity, and immunosuppressive outcomes of aplastic anemia in the Han population residing in southwest China ***(Zhao et al. 2018)***. Despite the absence of a significant association between the *IL23R* mRNA level and the SLEDAI score, Izati.AF *et al.* reported that the IL-$23R^+$ Th cells population was significantly associated with higher SLEDAI-2K scores but not with the immunological parameters ***(Izati et al. 2021).***

SLE patients are frequently treated with GC. However, a significant proportion of patients experience an exacerbation of their disease, needing more aggressive approaches such as methylprednisolone, and cyclophosphamide pulse therapy were used to provide temporary reductions

in disease activity. In our study, 22% of patients were under prednisolone or hydroxychloroquine as monotherapy with other GC or immunosuppressors, 10% took a combination of prednisolone and hydroxychloroquine together and 45% were under aggressive therapy. Our results show a significantly higher expression level of *IL23R* in SLE patients treated with hydroxychloroquine, cyclophosphamide, and methylprednisolone compared to all other groups. This suggests that *IL23R* mRNA expression level in SLE patients is affected by therapy. An earlier study has shown that dexamethasone decreases the expression of IL23p19 mRNA and protein levels which may affect the expression of IL23R in human monocytes ***(Palma et al. 2011)***. According to McGeachy *et al.*, the gene encoding *IL-23R* exhibits the highest upregulation when stimulated by *IL-23*, indicating the presence of a positive feedback loop for *IL-23* signaling via *IL-23R* ***(McGeachy et al. 2007)***. Taking together with our findings, these results suggest that *IL23R* is a later effector molecule in response to GC. To our knowledge, this is the first study to report an association between *IL23R* mRNA expression level and response to GC, antimalarial and immunosuppressive treatment in SLE patients.

These findings need to be validated on larger number of patients and controls, with an extended duration of follow-up.

In conclusion, we found that GRα mRNA expression has decreased in SLE patients compared to HC, suggesting a critical role of GRα in the pathophysiology of SLE disease, especially in skin damage. HDAC2 was associated with remission phase of SLE disease and IL23R was found to be associated with SLE clinical features and treatment. However, the relationship between these molecules and GC response is complex and appears to depend on the specific cell type and disease context and we hypothesise that none of the studied molecules could be potential prognostic markers to predict GC-insensitivity.

Key message

Despite the clinical association of GRα, HDAC2 and IL23R with SLE, none of these molecules could serve as a predictive biomarker for GC-insensitivity.

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Table S1: Primers used for qPCR Primer

Gene	Primers	product Length (bp)
GRα	F 5'- gaaggaaactccagccagaac -3' R 5'- ctgattggtgatgatttcagcta -3'	168
GRβ	F 5'- gaaggaaactccagccagaac -3' R 5'- tgacttattattgacaacgaagtgc -3'	204
HAT1	F 5' – gtgtacccagacaaaacccg -3' R 5'- ccgggaaaaacagggcaaat -3'	222
HDAC2	F 5'- gctactactacgacggtgatattgg -3' R 5'- ttcttcggcagtggctttatgg -3'	155
IL23R	F 5'- aaaacgtactggcagccttg-3' R 5'- agcccagaattccatgtgtc-3'	107
GAPDH	F: 5' gctctctgctcctcctgttc3' R: 5' cgcccaatacgaccaaatcc 3'	123

hGR: glucocorticoid receptor; HAT1: histone acetylase 1;HDAC2: histone deacetylase 2; IL23R: Interleukin-23 receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Table S2: Clinical and immunological features at the first diagnosis

Clinical features of SLE patients	
Symptoms	**(%)**
Malar rash	60
Photosensibility	43
Oral ulcers	4
Anemia	56
Arthritis	50
Polyarthralgia	66
Nephritis lupus	26
pleurisy	20
Raynaud syndrome	13
Pericarditis	36
Lymphopenia	53
Asthenia	46
Fever	60
Immunological features of SLE patients	
Auto-antibodies/components	**(%)**
ANA	100
Anti-dsDNA Abs	66
Anti-NUCLEOSOME Abs	50
Anti-CENTROMERE Abs	0
Anti-HISTONE Abs	36
Anti-Sm Abs	30
Anti-RNP Abs	53
Anti-SSA Abs	46
Anti-Ro-52 Abs	30
Anti-SSB Abs	23
Anti-Scl 70 Abs	3
Anti-Ribosome Abs	0
Anti-Mi 2 Abs	6
Anti-DFS 70 Abs	13
Low Complement 3 (C3) level	91
Low Complement 4 (C4) level	100
Rheumatoid factor	25

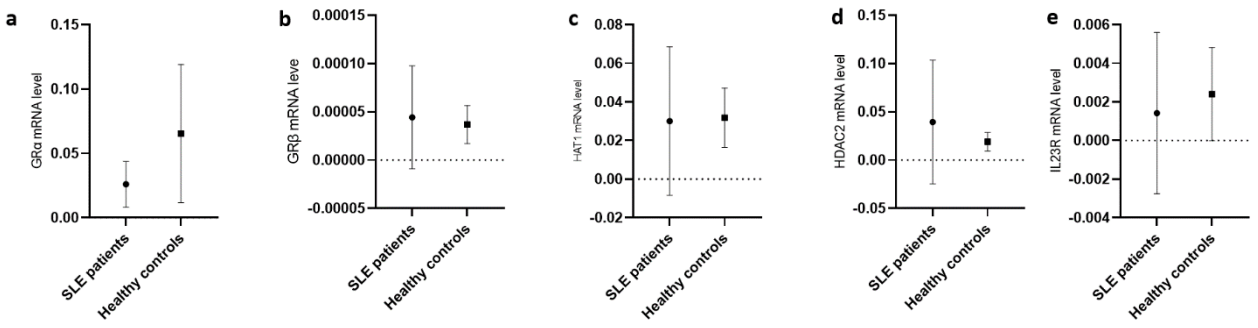

Figure S2: The overall expression of all studied genes (a) GRα mRNA expression level; (b) GRβ mRNA expression level; (c) HAT1 mRNA expression level; (d) HDAC2 mRNA expression level; (e) IL23R mRNA expression level in the 2 groups of SLE patients and the healthy controls.