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Hydroxychloroquine Suppresses Interferon-inducible Genes and B Cell Activating Factor in Patients With Incomplete and New-onset Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** Hydroxychloroquine (HCQ) is commonly used as first-line treatment for systemic lupus erythematosus (SLE). Interferon (IFN)-inducible gene expression, IFN-γ–induced protein 10 (IP-10) and B cell activating factor (BAFF) are early mediators in SLE. The purpose of this study was to analyze the effects of HCQ on these factors.

Methods. Patients with incomplete SLE (iSLE; antinuclear antibody titer ≥ 1:80, symptoms < 5 years, ≥ 1 objectified clinical American College of Rheumatology or SLE International Collaborating Clinics criteria), or new-onset, mild SLE were included when HCQ treatment was started for clinical reasons. Blood samples were taken at start and after 16 weeks. Three SLE-related IFN-inducible genes were measured in whole blood by real-time PCR, and an IFN score was calculated. Serum levels of IP-10 and BAFF were measured using ELISA.

Results. In total, 9 patients were included: 7 with iSLE and 2 with new-onset SLE. The median SLE Disease Activity Index (SLEDAI) was 4. After 16 weeks of treatment with HCQ, the expression of IFN-inducible genes decreased in 8 of 9 patients, and the IFN-3 score decreased significantly ($P = 0.012$). There was a trend towards lower IP-10 levels ($P = 0.055$), and a significant decrease in BAFF levels ($P = 0.023$).

Conclusion. HCQ suppresses IFN score and BAFF levels in patients with iSLE or new-onset SLE, and there is a trend towards lowering IP-10 levels. As these biomarkers are early mediators in SLE, this might support the hypothesis that HCQ could influence disease progression. However, prospective research with a larger sample size and longer follow-up is needed.

Key Indexing Terms: BAFF, hydroxychloroquine, interferon, IP10, lupus, SLE

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with heterogenous features of organ inflammation and immunologic aberrations with antinuclear antibodies (ANA) as a hallmark. Despite various treatment options, there is no cure, and damage develops in 77% of patients.[1]

Originally, the American College of Rheumatology (ACR) classification criteria were developed. In 2012 the SLE International Collaborating Clinics (SLICC) revised these criteria.[2,3] Recently, new European League Against Rheumatism (EULAR)/ACR classification criteria have been published in order to increase sensitivity and specificity.[4]

Hydroxychloroquine (HCQ) is the backbone of SLE treatment due to its effectiveness in preventing disease flares and improving survival.[5] This drug has been suggested to delay onset of SLE in patients with SLE symptoms, but who do not yet meet the classification criteria; this is a condition that we refer to as incomplete SLE (iSLE).[6] Up to 55% of patients with iSLE will progress to classified disease.[7]

One of the mechanisms of action of HCQ is the blockade of Toll-like receptor (TLR)-7 and TLR-9. The signaling of these receptors results in the production of interferon (IFN)-α and its effector cytokines. IFN-α is an important mediator in the pathogenesis of SLE. Elevated IFN-inducible gene expression—so-called IFN signature—is found in the majority of patients with SLE.[8] Interestingly, the presence of an IFN signature in patients with iSLE is associated with progression to SLE.[9] IFN-α stimulates production of IFN-γ–induced protein 10 (IP-10) and B cell activating factor (BAFF). These cytokines are increased not only in manifested SLE but also prior to SLE diagnosis.[10]

The purpose of this study was to analyze the effects of HCQ treatment on IFN-induced gene expression, serum IP-10, and BAFF levels in patients with iSLE and new-onset SLE.

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METHODS

Patients. This study was conducted as part of a prospective study on iSLE. Patients with iSLE (ANA titer ≥ 1:80, symptoms < 5 yrs, ≥ 1 objectified clinical ACR or SLICC criterion), or new-onset SLE (newly diagnosed SLE based on ACR 1997 or SLICC criteria, without serious organ involvement) were included when started on HCQ treatment as part of regular clinical care. In retrospect, ACR/EULAR sum score was calculated. Exclusion criteria were other connective tissue diseases, including rheumatoid arthritis. Blood samples were taken at start of treatment and after 4–6 months. Apart from nonsteroidal antiinflammatory drugs (NSAID), patients used no other immunosuppressive drugs (including corticosteroids). The SLE Disease Activity Index 2000 (SLEDAI-2K) score was assessed at both timepoints.[11] Blood samples of 22 healthy controls (HC) from a previous study (82% female, median age 45 yrs), were used as references for calculation of IFN scores.[12] The research protocol was approved by the Institutional Medical Ethical Committee (METc 2015/313). All subjects provided written informed consent.

Methods. Clinical data and standard laboratorial measurements were retrieved from electronic patient records. ANA were measured by indirect immunofluorescence technique using HEp2-cells as substrate. Anti-dsDNA antibodies and other autoantibodies were measured using the automated EliA assay (ENA CTD screen, ThermoFisher Scientific). The serum level of HCQ was measured by liquid chromatography–tandem mass spectrometry.

Whole blood samples were collected in PAXgene RNA tubes and stored at –20°C. After defrosting, RNA was isolated using PAXgene Blood RNA Kit (Cat No./ID:762164). The isolated RNA was reversely transcribed to cDNA and subsequently quantitatively analyzed using an Applied Biosystems QuantStudio Flex Real-Time PCR System. Expression of 3 commonly known SLE-related IFN-inducible genes were measured: *IFI44L*, *LY6E*, and *MX1*. Relative expression (RE) was calculated based on the cycle threshold (Ct) value related to expression of the housekeeping gene *GAPDH* as follows: $RE = 2^{-(Ct\ Test\ gene - Ct\ GAPDH)}$. The Ct value of *GAPDH* was confirmed to be stable. IFN score was calculated by summing up the log-transformed RE after normalization as follows: $\Sigma(RE_{subject} - Mean_{hc})/SD_{hc}$. All blood samples (before and after starting treatment) were tested simultaneously.

Levels of serum IP-10 and BAFF were measured by ELISA (Duoset, R&D Systems). High performance ELISA buffer (Sanquin) was used during serum incubation to prevent nonspecific reactions.

Statistics. Statistical tests were performed in GraphPad Prism (version 7.02). Differences between 2 timepoints at group levels were analyzed with Wilcoxon matched-pairs signed-rank test. Correlations were calculated using Spearman *r* test.

RESULTS

In total, 9 patients were included: 7 with iSLE and 2 with new-onset SLE (see Table 1 for characteristics). Median SLEDAI was 4 at the start of treatment. HCQ was indicated for synovitis

Table 1. Patient characteristics.

	1	2	3	4	5	6	7	8	9
Reason for HCQ start	Tendinitis	Arthritis	Arthritis	Arthritis	Arthritis	Arthritis	Arthritis	CLE	Arthritis
Sex	F	F	M	F	M	M	F	M	F
Age, yrs	29	26	68	55	56	41	30	37	59
Disease duration, mos	2	3	9	6	21	3	24	13	12
Simultaneous start of NSAID	No	Yes	Yes	No	No	Yes	Yes	No	Yes
No. ACR criteria	3	3	3	2	4	2	3	3	3
No. SLICC criteria	2	3	3	2	4	3	4	3	3
Sum score, EULAR/ACR criteria	17	13	12	6	16	8	9	10	8
Clinical criteria									
Skin	–	x	–	–	–	–	x	x	–
Ulcers	–	–	–	–	–	–	–	–	–
Synovitis	–	–	x	x	x	x	x	–	x
Hematologic	x	–	–	–	–	–	–	–	–
Serositis	–	–	–	–	x	–	–	–	–
Renal	–	–	–	–	–	–	–	–	–
Neurologic	–	–	–	–	–	–	–	–	–
Immunologic criteria									
ANA	x	x	x	x	x	x	x	x	x
Anti-dsDNA	x	–	x	–	–	–	–	x	–
Anti-SSA	x	–	–	x	–	–	–	x	–
Anti-Sm	–	x	–	–	–	–	–	–	–
Anti-RNP1	–	x	–	–	–	–	–	–	–
Low complement	–	–	–	–	–	–	x	–	–
Antiphospholipids	–	–	–	–	x	x	–	–	x
HCQ serum level, µg/L	628	147	342	564	315	233	258	129	496
Resolution of symptoms	No	No	No	No	Yes	Yes	No	Yes	Yes
SLEDAI before HCQ[specified]	0	4[a]	6[a,b]	4[a]	6[a,c]	4[a]	6[a,c]	2[d]	4[a]
SLEDAI after HCQ[specified]	0	1[e]	2[b]	4[a]	0	0	2[c]	2[d]	0

Specification of SLEDAI score: [a] arthritis (4 points); [b] anti-dsDNA (2 points); [c] low complement 3 or 4 (2 points); [d] skin rash (2 points); [e] leukopenia (1 point). ACR: American College of Rheumatology; ANA: antinuclear antibody; CLE: cutaneous lupus erythematosus; EULAR; European League Against Rheumatism; HCQ: hydroxychloroquine; NSAID: nonsteroidal antiinflammatory drug; SLICC: SLE International Collaborating Clinics; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

or arthritis in 8 patients and cutaneous lupus erythematosus in 1 patient. All patients had therapeutic levels of HCQ, although 2 patients had HCQ levels that were in the lower part of the therapeutic range.

After 4–6 months of HCQ treatment, the IFN-3 score decreased significantly (P = 0.01; Figure 1). At an individual level, relative expressions of all 3 IFN-inducible genes decreased in 8 of the 9 patients, which at group level reached significance in *MX1* (P = 0.05) and *LY6E* (P = 0.04), but not in *IFI44L* (P = 0.13). The single patient (subject no. 2), who showed an increasing IFN score, had iSLE, but developed SLE 15 months after starting HCQ. This patient had a HCQ serum level in the lower segment of the therapeutic range (147 μg/L). This patient later developed Sjögren syndrome. None of the other patients with iSLE developed new symptoms after median follow-up of 13 months (range 9–37 mos).

There was a trend towards lower IP-10 levels (P = 0.055), and a significant decrease in BAFF levels (P = 0.023) after starting treatment with HCQ. Of the 9 patients, 8 showed a decrease in levels of IP-10 and BAFF. Another subject (no. 9) showed increasing BAFF and IP-10 levels (Figure 2).

Median SLEDAI decreased significantly (P = 0.03; Figure 2), but the change in SLEDAI was not significantly correlated with change in IFN scores and levels of IP-10 and BAFF. (Supplementary Figure 1, available from the authors on request).

There was no significant change in numbers of leukocytes, lymphocytes, monocytes, neutrophils, levels of anti-dsDNA, complement, and total IgM or total IgG levels after starting HCQ treatment.

DISCUSSION

In patients with iSLE or early mild SLE, treatment with HCQ resulted in a significant decrease of whole-blood expression of IFN-inducible genes, as well as BAFF levels. Further, SLEDAI decreased significantly after treatment start with HCQ. However, a subjective relief of symptoms only occurred in 4 of the 9 patients. One patient had increasing IFN score, and 1 other patient had increasing levels of BAFF. Both these patients developed classified systemic immune disease. We are currently following iSLE patients in a prospective study, to provide more insight in the role of IFN, BAFF, and IP-10 in development of classified autoimmune disease.

There are a few publications on the influence of HCQ on IFN expression in patients with SLE, but the outcomes are not consistent. A longitudinal study in SLE patients showed no significant decrease in IFN-α2 after 6 months of HCQ use, although there was a strong correlation between the decrease in IFN-α2 and disease activity.[13] Olsen, *et al* performed a cross-sectional study in patients with iSLE and SLE, showing that iSLE patients taking HCQ had significantly lower IFN gene expression levels.[14] Notably, this difference was not found in patients with SLE. But in a recent cross-sectional study on IFN gene expression by our research group, there was no difference in IFN scores related to HCQ use in iSLE patients, nor in SLE.[12]

Two previous studies tested IP-10 serum levels with regard to HCQ use.[13,14] Both found no significant differences in IP-10 serum levels, similar to the current study.

Recently, a decrease in BAFF levels after starting treatment with HCQ has also been found in a large cross-sectional study in

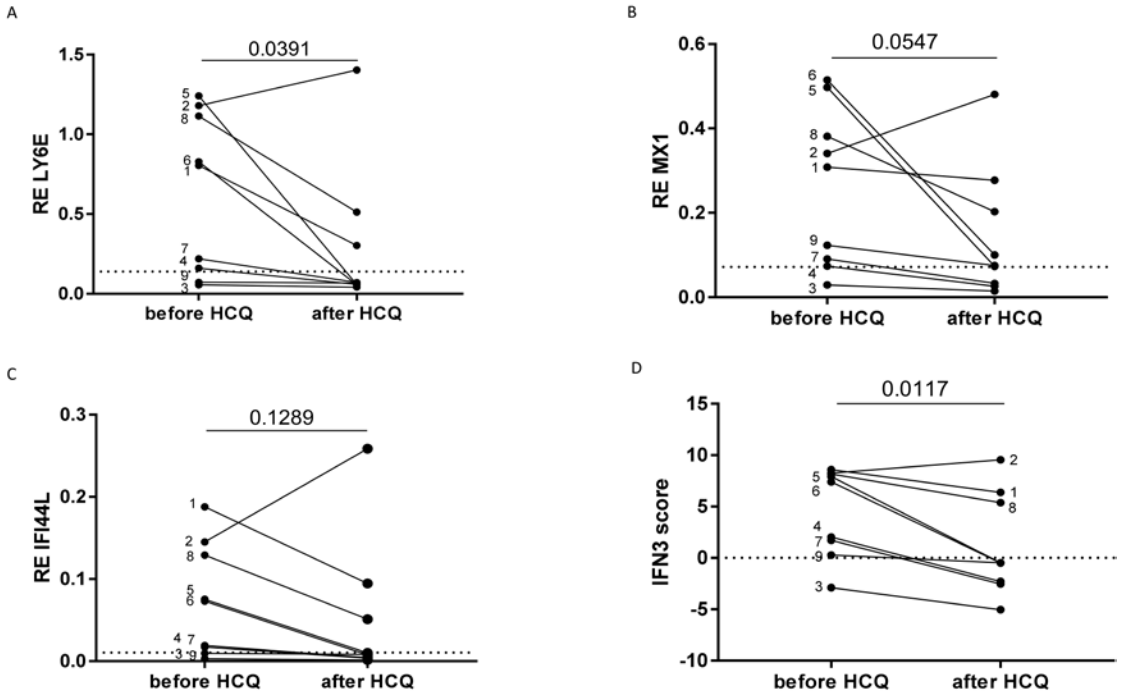

Figure 1. Expression of IFN-inducible gene expression upon treatment with hydroxychloroquine. Relative expression (RE) of IFN-inducible genes (A) *LY6E*, (B) *MX1*, (C) *IFI44L*, and (D) IFN-3 score, before and after treatment with HCQ. The level of significance (P value) is depicted. All patients are labeled by their subject numbers (see Table 1.) The dotted line represents the mean ± SD of the healthy controls. HCQ: hydroxychloroquine; IFN: interferon.

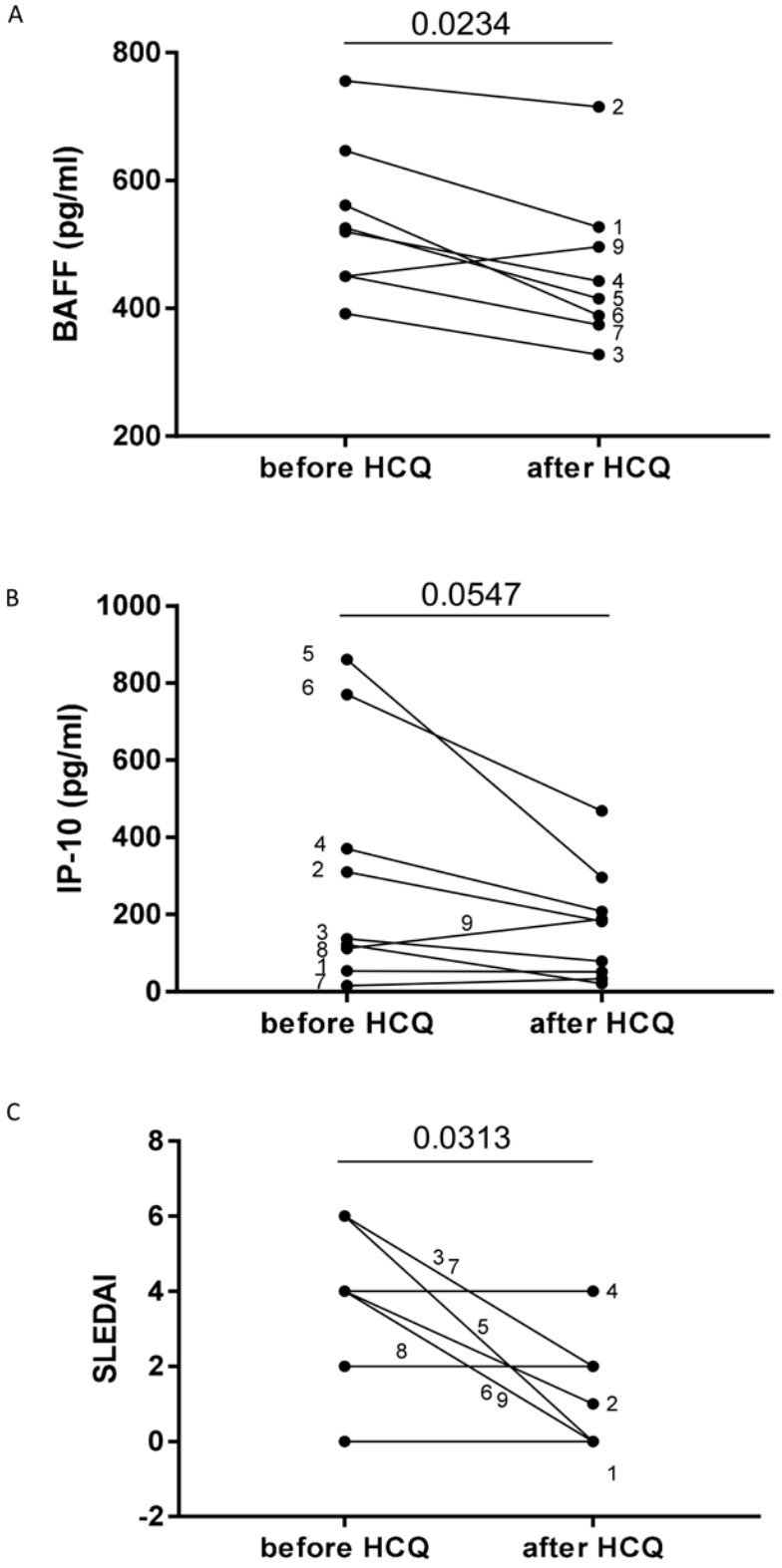

Figure 2. Change of IFN-related mediators and SLEDAI after treatment with HCQ. Levels of (A) B cell activating factor (BAFF), (B) IFN-γ–induced protein 10 (IP-10), and (C) SLEDAI, before and after treatment with HCQ. The level of significance (*P* value) is depicted. All patients are labelled by their subject numbers (see Table 1.) HCQ: hydroxychloroquine; IFN: interferon; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

SLE.[15] As BAFF levels are associated with higher disease activity, inhibition of BAFF production might be one of the potential mechanisms of action of HCQ.

The main limitation of this study is the small sample size. Remarkably, 4 of the 9 patients are males. The sample size, however, was too small to be able to draw any conclusions about the male predominance in this study group. Second, this patient subgroup is not representative of all patients with SLE, as patients had incomplete or mild forms of the disease. On the other hand, inclusion of patients with iSLE or new-onset SLE is a strong point, as these patients are not using other immunosuppressive drugs, allowing the evaluation of the effect of using HCQ alone. Also, therapy adherence was ensured by measuring serum levels of the drug. Still, bias of the effect of simultaneous start of NSAID cannot be excluded; however, the mechanism of this drug would not explain an effect on IFN-related mediators.

In conclusion, this small study provides evidence for HCQ suppressing IFN-inducible gene expression and BAFF-levels in patients with iSLE or new-onset mild SLE. Further, there is a trend towards lower IP-10 levels after starting HCQ. As these mediators are increased in patients who later develop SLE, it would be interesting to investigate whether starting HCQ in this early phase delays disease progression in iSLE and new-onset SLE.[16]

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