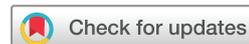# Influence of hydroxychloroquine blood levels on adhesion molecules associated with endothelial dysfunction in patients with systemic lupus erythematosus

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ABSTRACT

Background Patients with SLE have an endothelial dysfunction (ED), which is considered the earliest marker of cardiovascular (CV) disease. Endothelial cell activation induced by proinflammatory cytokines is defined by the endothelial expression of cell-surface adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin. The aim of this study was to investigate whether serum endothelial adhesion molecule levels are influenced by blood hydroxychloroquine (HCQ) levels in SLE.

Methods Consecutive patients with SLE taking a stable dose of HCQ were investigated. At study entry and 6 months later HCQ blood levels were quantified by tandem mass spectrometry. Serum levels of P-selectin, E-selectin, ICAM-1 and VCAM-1 were also measured using a Luminex 200 instrument. Comparison of endothelial soluble adhesion molecules in groups with different HCQ blood levels was performed by t-test.

Results 83 patients with SLE were enrolled. Correlation were demonstrated between mean blood HCQ concentrations and endothelial soluble adhesion molecules (E-selectin, ICAM-1 and VCAM-1). Moreover, serum levels of ICAM-1 and VCAM-1 were significantly lower in the patients with SLE with HCQ blood levels >500 ng/mL (83.67±52.8 ng/mL vs 158.81±125.1 ng/mL and 8.9±2.2 ng/mL vs 10.4±2.3 ng/mL). Serum levels of E-selectin were nearly significantly lower in the patients with SLE with HCQ blood levels >500 ng/mL (64.7±30.2 ng/mL vs 71.6±42.2 ng/mL, p=0.06). No significant difference in concentration of P-selectin was detected.

Conclusions In the present study, there was a trend towards higher adhesion molecules levels with lower HCQ blood levels in patients with SLE. Further longitudinal studies will determine whether changes in endothelial biomarkers reflect decreased clinical CV events.

INTRODUCTION

Despite the improvement in survival over the past decades, SLE is still characterised by increased mortality due to cardiovascular diseases (CVD).[1] The high risk of CVD in SLE is only partially explained by an increased prevalence of traditional CV risk factors;

Key messages

What is already known about this subject?
► Endothelial function appears to be affected by SLE, potentially contributing to the increased cardiovascular (CV) risk observed in these patients.
► Endothelial dysfunction in these patients is characterised by increased expression and release of adhesion molecules.

What does this study add?
► In this study, therapeutic blood hydroxychloroquine (HCQ) levels were associated with decreased adhesion molecules levels in SLE.

How might this impact on clinical practice or future developments?
► These findings further support the previous observations of the potential beneficial effect of HCQ in preventing CV disease in SLE.

SLE-related factors also contributing to the risk.[2] SLE-associated systemic inflammation may impair endothelium functionality, leading to atherosclerosis development and progression.[3]

Endothelial dysfunction (ED) is recognised as an early marker of atherosclerosis and is characterised by the inability of the endothelium to appropriately modulate blood flow due to a reduced bioavailability of endothelium-derived vasodilators, such as nitric oxide and a concomitant increased reactive oxygen species generation.[4] Various studies have shown that patients with SLE have an ED.[5] In addition to ED, another important feature of atherosclerotic vessels in SLE is endothelial cell activation.[6] The expression of adhesion molecules on activated endothelial cells is an important step in the initiation and progression of atherosclerosis. At the beginning of atherosclerotic plaque formation, endothelial cells show increased expression

and release of adhesion molecules, including E-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These molecules are released in response to stimuli of inflammatory cytokines and promote cell-cell and cell-extracellular matrix adhesion, leading to foam cell accumulation on the subendothelial space.[7] In particular, the endothelial selectins (E-selectin, P-selectin) mediate the initial weak interaction that allows leukocytes to roll along the vessel wall, while adhesion molecules of the immunoglobulin superfamily (ICAM-1 and VCAM-1) on endothelial cells mediate the second tight adhesion. In vitro studies have shown that the serum concentrations of soluble adhesion molecules correlate with the expression of these molecules on the endothelial cell surface.[8] Elevated concentrations of circulating soluble adhesion molecules were closely linked to the development of atherosclerosis and predict CV events.[8–11] Interestingly, ED is a reversible alteration, thus representing a potentially attractive target for preventive intervention.

Hydroxychloroquine (HCQ) remains the first-line treatment for patients with SLE owing to its safety profile and multiple beneficial effects, including control of disease activity, reduction of damage accrual and improvement of survival.[12–15] Moreover, HCQ has demonstrated to reduce serum cholesterol and mean glucose levels.[16 17] We recently conducted a long-term study which demonstrated the HCQ-related benefits in the primary thromboprophylaxis in patients with SLE.[18 19] A beneficial effect of HCQ on vascular reactivity indices has also been reported in lupus murine models.[20 21] Based on these evidences, we hypothesised that HCQ could restore ED. Therefore, the present study was designed to analyse the effect of HCQ blood levels on the concentrations of soluble adhesion molecules (ICAM-1, VCAM-1, E-selectin and P-selectin).

MATERIALS AND METHODS

Study population

Caucasian patients with SLE prospectively followed at our lupus clinic were eligible for inclusion in the study if they met the SLE American College of Rheumatology classification criteria[22] and were on treatment with a stable dose of oral HCQ during the previous 6 months. To avoid the effect of active inflammation on ED, we included patients in remission according to the Definitions of Remission in SLE criteria, for at least 1 year.[23]

The exclusion criteria included other autoimmune diseases, antiphospholipid antibody syndrome, preexisting CVD and currently taking medications that influence the endothelial function marker level, such as ACE inhibitor or statin. Data collected about each patient from admission to throughout follow-up include basic demographic characteristics (gender, date of birth, age at SLE onset, ethnicity), disease duration, disease manifestations, current and previous therapies, damage accrual and laboratory investigations, family history of vascular disease (ischaemic heart disease, stroke and peripheral vascular disease), menopausal status. At each visit, physical examination, treatment regimen, Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) and SLICC Damage Index (SDI),[24 25] adverse events and laboratory testing, including C3 and C4 levels and antidouble-stranded DNA antibodies were recorded.

HCQ extraction from whole blood and definition of non-adherence

Consecutive patients with SLE taking a stable dose of HCQ for at least 6 months were investigated. Patients were prescribed HCQ not exceeding 6.5 mg/kg (maximum 400 mg/day). At study entry (T0) and 6 months later (T6), a blood venous sample was taken to measure whole blood concentration of HCQ. If the patient had to discontinue HCQ use due to retinopathy or intolerance, was excluded. HCQ levels were serially quantified from EDTA whole blood by liquid chromatography-tandem mass spectrometry as previously described.[26] A mean HCQ value for each patient was then calculated. This study design was chosen as there are individual variations in HCQ bioavailability that are considered secondary to pharmacokinetic and pharmacodynamics factors, which are as yet poorly understood. The therapeutic range was 500–2000 ng/mL. This was chosen as our therapeutic range based on a review of the available literature. The patients were divided according to their blood level. Levels <100 ng/mL were considered to be consistent with complete non-adherence. Levels of 100–500 ng/mL were considered partially adherent, between 500 and 2000 ng/mL were therapeutic and >2000 ng/mL were considered supratherapeutic.[27]

Serum marker quantification

We then evaluated the serum levels of adhesion molecules (ICAM-1, VCAM-1, E-selectin and P-selectin). Peripheral blood was obtained at enrolment by venipuncture. Serum was separated by centrifugation, aliquoted and stored at −20°C. All analytes were measured by single or multiple suspension fluorescence-based immunoassay (Merck Millipore, Billerica, Massachusetts, USA) using a Luminex 200 instrument (Luminex, Austin, Texas, USA). Concentrations were expressed as ng/mL.

Statistical analysis

Continuous data were expressed as mean±SD or median with range and compared by the unpaired Student's t-test or the Mann-Whitney U test as appropriate. Categorical data were analysed by the Fisher's exact test and the χ^2 test, respectively. Correlation analysis between two variables was performed using Spearman's rank correlation. The grading of correlation coefficients (r) can vary, but for the purposes of this study 0.2–0.3=weak correlation, 0.3–0.7=moderate correlation and 0.7–1.0=strong correlation. For statistical analysis, the patients were divided according to mean HCQ blood levels (between baseline and follow-up). We compared the HCQ groups with respect to serum adhesions markers levels at baseline

Table 1 Clinical, demographic and laboratory features of 83 patients enrolled*

	All patients (n=83)	HCQ <100 ng/mL (n=24)	HCQ ≥100 ng/mL (n=59)	P value
Sex, female	79 (95)	22 (92)	57 (97)	0.575
Age, years mean±SD	41±11	39±11	42±11	0.772
Disease duration, years median (range)	15 (2–37)	17 (2–29)	13 (2–37)	0.692
SLICC Damage Index median (range)	0 (0–3)	0 (0–1)	0 (0–3)	0.385
Current smokers	31 (37)	9 (37)	22 (38)	0.816
Body mass index (kg/m^2) mean±SD	25±5	24±4	26±5	0.828
Estimated creatinine clearance, mL/min mean±SD	89±24	94±22	86±24	0.129
HCQ prescribed dose/weight (mg/kg) mean±SD	5.3±1.2	5.4±1.2	5.4±1.2	0.71
HCQ ng/mL median (range)	327 (0–4003)	0 (0–99)	546.1 (101–4003)	<0.0001
DCQ ng/mL median (range)	47 (0–650)	0 (0–11)	79 (11–650)	<0.0001
Additional treatment				
Immunosuppressors	23 (28)	13 (54)	10 (17)	0.001
Azathioprine	10 (12)	5 (22)	5 (8)	
Mycophenolate mofetil	6 (7)	3 (12)	3 (5)	
Methotrexate	5 (6)	4 (17)	1 (2)	
Ciclosporin	2 (2)	1 (4)	1 (2)	
Glucocorticoids	40 (48)	11 (46)	29 (52)	0.974

*If not otherwise specified, the values are the number (%) of patients.
DCQ, whole blood concentration of desethylchloroquine; HCQ, whole blood concentration of hydroxychloroquine; SLICC, Systemic Lupus International Collaborating Clinics.

and assessed significance using t-test. P values <0.05 were considered statistically significant. MedCalc software, V.15.4, was used for all analyses.

RESULTS
Demographic, clinical and laboratory features of patients
A total of 83 consecutive patients with SLE fulfilled the inclusion criteria and was enrolled in the study after giving written informed consent. Clinical and therapeutic features of the patients enrolled are summarised in table 1.

They were Caucasian, mostly female (95%), with a mean age of 41 years, a median disease duration of 15 years (range 2–37 years) and a median SDI of 0 (range 0–3). They were treated with HCQ prescribed at a stable oral dose of 400 or 200 mg/day for at least 6 months (the mean±SD daily HCQ dose was 346±72). The mean±SD dose of HCQ per weight prescribed was 5.3±1.2 mg/kg. The median HCQ blood concentration was 327 ng/mL (range 0–4003 ng/mL). There was no correlation between the prescribed HCQ dose in mg/kg (through the range of clinical use of 4.5–6.5 mg/kg/day) and the HCQ blood levels (r=−0.08, p>0.05). We stratified patients according to their blood HCQ levels. Among the 83 patients, 24 (29%) had values indicative of poor therapeutic adherence, reflected by at least HCQ blood levels ≤100 ng/mL or undetectable blood HCQ concentration. Additional 37 (44%) patients showed HCQ concentration <500 ng/mL in at least a visit. The proportion of

patients with mean HCQ levels 100–500 (subtherapeutic) and >500 ng/mL (therapeutic) was 30% and 41%, respectively. There were no statistically significant differences seen in demographic and clinical features between patients with therapeutic levels and non-adherents, except for immunosuppressants use (table 1). Among the 59 patients with mean whole HCQ blood levels in therapeutic range, 12 patients had a mean level >1.000 ng/mL. Despite appropriate weight-based dosing, a patient was above the therapeutic range (>2000 ng/mL).

Serum adhesion molecules and relationship with HCQ blood levels
All investigated factors (ICAM-1, VCAM-1, P-selectin, E-selectin) were detectable in sera from patients with SLE. In this regard, correlations between mean HCQ blood levels and serum adhesion molecules were performed. The analysis of the whole cohort showed that correlation coefficients ranged from −0.28 (p=0.03) for E-selectin to −0.38 (p=0.005) for ICAM and to −0.45 (p=0.001) for VCAM. Since these results can be influenced by the non-therapeutic HCQ whole blood levels found in non-adherent patients (HCQ whole blood levels ≤100 ng/mL), we excluded such patients from the analysis. The analysis of the remaining 59 patients showed that correlation coefficients ranged from −0.26 (p=0.04) for E-selectin to −0.36 (p=0.005) for ICAM and to −0.41 (p=0.001) for VCAM. Likewise, no correlation between serum P-selectin and mean blood levels of HCQ were found (data
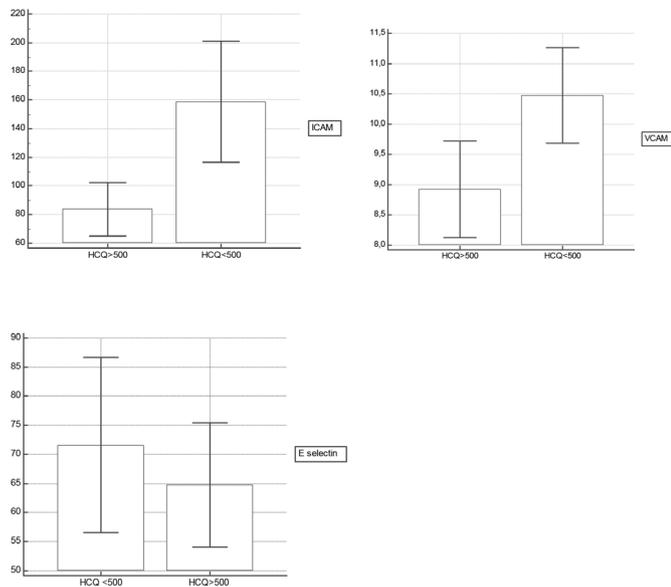
Figure 1 Mean of soluble intercellular adhesion molecule-1 (ICAM-1), soluble vascular cell adhesion molecule-1 (VCAM-1) and E-selectin levels, with difference between the two hydroxychloroquine (HCQ) groups (mean HCQ blood levels >500 ng/mL or <500 ng/mL).

not shown). We then compared each HCQ group with respect to serum adhesion molecules levels (figure 1). Serum levels of ICAM-1 and VCAM-1 were significantly lower in the patients with SLE with mean HCQ blood levels >500 ng/mL (respectively: 83.67±52.8 ng/mL (65.2–102.1) vs 158.81±125.1 ng/mL (116.4–201.1), p=0.001 and 10.4±2.3 ng/mL (9.6–11.2) vs 8.9±2.2 ng/mL (8.1–9.7), p=0.006). Serum levels of E-selectin were nearly significantly lower in the patients with SLE with mean HCQ blood levels >500 ng/mL (64.7±30.2 ng/mL (54.1–75.4) vs 71.6±42.2 ng/mL (56.6–86.5), p=0.06). No significant difference in concentration of P-selectin was detected in patients with SLE.

Disease duration, sex, age, body mass index, antiphopholipid antibodies (aPL), postmenopausal status did not correlate the serum profile of the investigated soluble factors in patients with SLE.

DISCUSSION

To our knowledge, this is the first study to evaluate the effect of HCQ on serum biomarkers of endothelial activation in patients with SLE. HCQ represents a milestone in the treatment of patients with SLE. In recent years, HCQ therapy has been associated with positive effect on traditional CV risk factors and a reduction of CV morbidity and mortality in SLE.[18 19] Moreover, maintaining an average HCQ whole blood level >1.068 ng/mL significantly reduced the risk of thrombosis in SLE, based on data from 739 patients of the John Hopkins cohort.[28] However, it was still unclear if the protective CV effect of HCQ is an indirect result of better control of disease activity or a direct effect of HCQ on the endothelium. It is known that after activation by proinflammatory cytokines, adhesion

molecules are shed from the surface of endothelial cells. For this reason, these soluble adhesion molecules may be useful as markers of endothelial activation.[8] This study shows that higher mean HCQ whole blood levels were associated with lower levels of soluble adhesion molecules. In particular, we found lower serum levels of E-selectin, soluble VCAM-1 (sVCAM-1) and soluble ICAM-1 (sICAM-1) in patients with mean HCQ blood levels in therapeutic range.

In our study, the role of HCQ in improving ED was not linked to a better control of the disease, as disease activity in both groups did not differ significantly. The present data confirm in humans the previous demonstration of the direct effect of HCQ in lupus mouse models on endothelium,[20] where endothelium-dependent vasodilator responses were normalised by HCQ. Data from studies previously published in Rheumatoid Arthritis (RA) patients revealed similar results.[29]

Moreover, in this study we confirmed the previous findings that adherence to medications is a problem in patients with SLE.[30–33] Our analysis demonstrates that up to 30% of our patients with inactive SLE did not take their HCQ as prescribed. Previous studies have shown that the rate of non-adherence assessed by HCQ blood measurement is highly variable, ranging from 7% up to 83%.[33 34] We pointed out that 24 (29%) had undetectable blood HCQ levels and additional 37 (44%) patients showed a HCQ concentration <500 ng/mL in a visit, although it is possible that in this group there may be individuals who, due to genetic differences in HCQ metabolism, are adherent but achieve lower blood concentrations. In our study, the rate of non-adherent patients decreased at the second sampling as only about 10% of patients had very low levels of blood HCQ. This is similar to the reported literature in SLE and monitoring HCQ blood levels can be important to improve medication adherence in patients with lupus.[34 35] Moreover, in our study there was no correlation between the prescribed HCQ dose and the HCQ blood levels. Our data clearly indicate the need for personalised HCQ dosing approaches beyond empirical dosing recommendation through routinely HCQ blood level measurements.

Our study has several limitations. The endothelial biomarkers that we used were in circulating form, which have less important biological function than the form that is present on the surface of endothelial cells. Moreover, the ED improvement was characterised by lowering of E-selectin, sVCAM-1 and sICAM-1 levels in HCQ group in therapeutic range. However, these results were not confirmed prospectively and were not followed by a significant decrease in P-selectin levels. However, E-selectin may be more specific in reflecting the atherosclerotic burden compared with P-selectin, as is expressed exclusively by activated vascular endothelium, whereas P-selectin also mediates platelet-neutrophil interactions.[36] Another limitation is that the targeted threshold HCQ levels differed between studies. Some studies used <200 ng/mL as the threshold HCQ blood level to

identify severe non-adherence in patients treated with 400 mg/day of HCQ.[31] We used a cut-off of 100 ng/mL, which is more stringent to exclude any possible interference with factors known to influence HCQ blood concentration. Similarly, other studies used HCQ levels >500 ng/mL as a therapeutic threshold.[33] Additional limitations include the single site and small sample size.

In conclusion, our study showed significant reduction in endothelial biomarkers in patients with SLE with therapeutic HCQ blood levels but there is no proof of causality and further research is needed to find molecular mechanisms that can explain if and how HCQ can improve ED in patients with SLE.

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