

Hydroxychloroquine in Systemic Lupus Erythematosus: From Molecular Mechanisms to Biomarkers and Systemic Outcomes

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www.sjmars.com || Vol. 5 No. 4 (2026): August Issue

Date of Submission: 16-08-2026

Date of Acceptance: 23-08-2026

Date of Publication: 30-08-2026

ABSTRACT

Background: Systemic Lupus Erythematosus (SLE) is a chronic, relapsing, multi-system autoimmune disorder. It involves loss of tolerance to nuclear antigens, immune-complex formation, complement activation and the presence of a type I interferon signature.

Importance of HCQ: One of the cornerstones of SLE therapy is Hydroxychloroquine (HCQ). It is currently recommended for most patients, except in cases where this is contraindicated.

Molecular and biochemical mechanisms: The HCQ is a weak diprotic base. It targets to acidic lysosomes and endosomes and increases their internal pH. This affects the signalling of endosomal Toll-like receptor 7 and 9 and reduces production of downstream MyD88, interferon regulatory factor 7, and type I interferon. HCQ may also directly interact with nucleic acids and prevent them from being recognized by these receptors.

Biomarker and systemic effects: The observational evidence links use of HCQ with a decrease in disease activity, less flares, less exposure to glucocorticoids and improved survival. At therapeutic concentrations, HCQ has been associated with a beneficial effect on glucose and lipid metabolism and with decreased thrombosis. Other benefits reported are renal and obstetric.

Evidence gaps: Many of the systemic benefits are based on observational cohorts. Causal inference is limited by confounding by indication and adherence bias.

Conclusion: The mechanisms of action of HCQ seem to involve all the immunological, inflammatory, metabolic, and vascular pathways, which are interconnected. The clinical biochemistry tools will enable these effects to be translated into biomarkers, monitoring, and ultimately personalised therapy.

Keywords- hydroxychloroquine; systemic lupus erythematosus; Toll-like receptors; type I interferon; biomarkers; therapeutic drug monitoring; lupus nephritis; thrombosis; retinopathy.

I. INTRODUCTION

SLE is the prototype of a systemic autoimmune disease. It may involve the skin, joints, kidneys, blood, serous membranes or the nervous system. It is a disease that has remissions and flares. Damage is caused by disease activity and treatment over the years [1]. In SLE the clearance of apoptotic and NETotic material is inefficient, resulting in immunological core: loss of self-tolerance to nucleic acids and associated proteins. Nuclear antigens remain, and thus are presented to the immune system. B and T cells are multiply. Autoantibodies are developed against double-stranded DNA, nucleosomes and ribonucleoproteins [1,3]. These autoantibodies bind their antigens and form immune complexes. The complexes get deposited in the tissues and activate the complement. Complement proteins C3 and C4 are used up during active disease. Immune complexes also bind innate sensors. The endosomal Toll-like receptor (TLR) family (TLR7 and TLR9) detects the nucleic-acid cargo. This activates production of type I interferon and a self-amplifying inflammatory loop [1,3]. Another

distinctive feature is the dysregulation of cytokines. There is often an increase in the levels of type 1 interferon, interleukin-6 and tumour necrosis factor. Endothelium is damaged through chronic inflammation. It also disrupts lipid & glucose metabolism. These alterations increase the cardiovascular and thrombotic risk in SLE [1,14]. Clinical implications are wide-ranging. Big complications include lupus nephritis, cytopenias, serositis, neuropsychiatric disease, and accelerated atherosclerosis. It is hard to manage in the long-term. Disease is heterogeneous. The glucocorticoids regulate activity, but cause metabolic, skeletal, and infective damage over time [2,6].

Hydroxychloroquine is a drug with proven benefits in this context. It is advised in most cases of SLE (unless there is a specific contraindication) [2]. Antimalarials are well tolerated and decrease flares and disease activity. For this reason the use of HCQ is considered background therapy, not a recommended option as an add-on [2,5].

This review suggests that HCQ should be considered not only as an activity control. It targets a molecular target upstream in the innate pathway leading to interferon signature [3,4]. If this is the case, then these effects would be apparent in biochemical intermediates and lab-based biomarkers. They can also lead to systemic effects like survival, renal preservation and thrombosis [8].

Review objective:

This review aims to clinically investigate, from the clinical biochemistry perspective, the impact of HCQ on molecular and biochemical pathways in SLE, the potential for these changes to be quantified in measured laboratory markers and the potential for these changes to contribute to systemic and long-term outcomes of SLE while separating established effects from proposed mechanisms and ambiguous associations.

II. SLE PATHOPHYSIOLOGY FROM A CLINICAL BIOCHEMISTRY PERSPECTIVE

There are several points in SLE where tolerance is lost. There is incomplete central and peripheral deletion of autoreactive lymphocytes. Function of the regulatory T cell is compromised. Excessive help is given to autoreactive B cells. The net effect is the continued production of anti-nuclear autoantibodies [1,22]. One of the most important upstream events is the failure to remove dead cells. Nuclear material is released by apoptotic bodies and NETs. This material is not optimally cleared in SLE. Thus, self nucleic acids end up in endosomes and extracellular space. They are made available as autoantigens and as innate ligands [1,3]. Two arms of recognition of these nuclear antigens run parallel. Antigen-presenting cells (APCs) process and present peptides to T cells in the adaptive arm. Nuclear acids are directly sensed by pattern-recognition receptors in innate arm. Both arms are directed towards interferon-induced inflammation [1,3]. Autoantibody formation is the serological signature of the disease. Almost all individuals have antinuclear antibodies. Anti-double-stranded DNA antibodies are more specific and tend to reflect activity. Anti-Smith antibodies are anti-nuclear factors, but they're less dynamic. These antibodies can be measured and are clinically relevant, and some of them are included in the 2019 EULAR/ACR classification criteria [1,7,24]. The next biochemical layer are immune complexes. Circulating and in-situ complexes are formed by antibody (ab) to nuclear antigen (na). The complexes deposit in the glomeruli, the skin and the walls of vessels. There, they cause local inflammation and tissue damage, most prominently in cases of lupus nephritis [1,17,33]. Immune-complex deposition is followed by complement activation and complement consumption. The classical pathway is activated. C3, C4 are consumed during active disease. The anti-double-stranded DNA and C3, C4 are used clinically as activity markers, the levels of which are low in those affected. It is amplified by innate immune activation. Plasmacytoid dendritic cells and B cells take up immune complexes with RNA or DNA. They are located in the acidified endosome, where they encounter Toll-like receptor 7 and Toll-like receptor 9. TLR7 recognizes ssRNA. Unmethylated CpG DNA is recognized by TLR9 [3,4]. Signalling then follows a specific pathway. Ligated TLR7 and TLR9 bind to MyD88. MyD88 forms a signalling complex that activates interferon regulatory factor 7 and nuclear factor kappa B. Type I interferon genes are transcribed by IRF7. Pro-inflammatory cytokines are promoted by the activation of nuclear factor kappa B [3,4]. Type I interferon plays a key role in lupus pathogenesis. It helps to mature dendritic cells, produces autoantibodies and maintains the inflammatory loop. Most patients with active disease have a blood interferon signature. This signature is a biomarker of the pathway which HCQ is believed to act on [1,3,23]. Interferon is not the only cytokine that is subject to dysregulation. Interleukin-6, tumour necrosis factor, and interleukin-17 help to cause inflammation. There are intrinsic signalling abnormalities in B and T cells. These include calcium flux changes and metabolic reprogramming in favour of activation [1]. The biochemically important vascular and metabolic consequences are. Endothelial dysfunction and oxidative stress are caused by chronic inflammation. It alters lipid metabolism to a proatherogenic profile. It promotes insulin resistance. These contribute to the increased cardiovascular and thrombotic risk associated with SLE [1,14].

2.1 Major biochemical and immunological abnormalities in SLE and their biomarkers

Systemic lupus erythematosus (SLE) is a set of sequential immunological and biochemical abnormalities, each of which may correlate with some laboratory markers. The process starts with loss of immune tolerance to nuclear antigens, which is linked to the impaired clearance of apoptotic cells and neutrophil extracellular traps (NETs) that lead to the accumulation of nuclear components and the triggering of auto-reactive immune responses. The antigen/antibody reaction process is widely recognized as the presence of the antinuclear antibody (ANA) test [1,3]. This loss of tolerance leads to the production of pathogenic autoantibodies, especially anti-dsDNA and anti-nucleosome antibodies, which can be quantified

and measured as anti-dsDNA and anti-nucleosome antibodies, respectively; of these, anti-dsDNA has specific clinical significance in SLE [1,7]. Other disease specific auto-antibodies include anti-Sm, anti-U1RNP, anti-Ro/SSA, and anti-La/SSB antibodies, of which anti-Sm is most specific to SLE [1]. These autoantibodies bind to nuclear antigens and generate immune complexes which can be found in the blood and then deposited in tissues, especially in the renal glomeruli, the skin and vascular walls. The presence of circulating immune complexes can be indirect evidence of it, and tissue deposition can be seen via biopsy [1,17]. This leads to immune-complex deposition and, subsequently, to an excess of the consumption of complement via the classical pathway. This makes it possible to use reduced serum levels of both C3 and C4, as well as elevated levels of complement activation products, as laboratory markers of complement activation and disease activity [1,2]. Nucleic-acid containing immune complexes can be ingested into endosomal compartments where recognition of nucleic acids by the Toll-like receptors 7 and 9 (TLR7 and TLR9) triggers the production of type I interferon. Type I interferon (IFN) signatures or interferon (IFN)-inducible gene-expression profiles can be used to assess this pathway [3,4]. The abnormal signalling leads to dysregulation of cytokines, including type I interferons, interleukin-6 (IL-6) and tumor necrosis factor (TNF), which can be assessed by measuring these cytokines in the circulation or by measuring interferon-responsive transcripts [1]. Systemic inflammation is a result of the immune activation that occurs over time and is often seen in an elevated erythrocyte sedimentation rate (ESR). Unlike C-reactive protein (CRP), however, it may only increase slightly during a SLE flare-up and so a large elevation should cause investigation for a concurrent infection [1]. Renal immune injury is one of the key clinical consequences, where immune-complex deposition in the glomerula leads to loss of filtration barrier function and results in measurable changes like proteinuria, urine protein-to-creatinine ratio (UPC), serum creatinine and estimated glomerular filtration rate (eGFR) [2,17]. Last but not least, chronic systemic inflammation may lead to endothelial and metabolic alterations, such as oxidative stress, a proatherogenic alteration of the lipid metabolism, and insulin resistance. The lipid profile, the fasting glucose level, the glycated hemoglobin (HbA1c) and markers of endothelial activation can be used to assess these abnormalities, providing laboratory evidence of the wide-spread effects of SLE on the vascular and metabolic system [9,12].

III. HYDROXYCHLOROQUINE: PHARMACOLOGICAL AND BIOCHEMICAL BASIS

Hydroxychloroquine is a 4-aminoquinoline drug. It is hydroxylated derivative of chloroquine. It is a weak diprotic base that is lipophilic. These physicochemical characteristics influence the location of the drug within cells, as well as the effects of the drug within cells [3,4]. This basicity is followed by cellular uptake. The non-protonated form of the drug can pass through membranes easily. It then goes into acidic compartments like lysosomes and endosomes. There it undergoes protonation and gets trapped. This ion trapping results in very high intra-cell and intra-organelle to plasma [3,4]. Thus, the drug preferentially targets the lysosomal/endosomal system. Most proposed mechanisms are based on its accumulation there. The long terminal half-life of HCQ (weeks) and its slow onset of clinical effect are likely related to its wide distribution into tissues [3,6]. Its main biochemical effect is an increase in intra-organelle pH. In the acidic environment of endosomes/lysosomes, protonated HCQ neutralizes the acidic content. The pH of the compartment increases. As such, there are many pH sensitive processes inside these organelles that are affected [3,4]. One of these affected processes is the processing and presentation of antigens. There are pH-sensitive steps in the processes of proteolysis of antigens and loading onto major histocompatibility complex (MHC) class II molecules. But HCQ can lower autoantigen presentation efficiency by elevating endosomal pH. This is a likely factor which contributes to the decrease of T-cell help, albeit less well characterised in humans [3,4]. The action which is best characterized is the inhibition of endosomal Toll-like receptors. TLR7 and TLR9 needs acidic pH to bind nucleic-acid ligands and to signal. HCQ reduces this recognition by neutralizing the pH in endosomes. A complementary mechanism has arisen out of the structural work. Both HCQ and chloroquine are expected to directly bind to nucleic acids and inhibit the binding to the receptor [3]. The downstream signalling consequences are a logical consequence. Modulation of engagement of TLR7/TLR9 leads to decreased activation of MyD88-dependent signalling. This reduces activation of interferon regulatory factor 7 and nuclear factor kappa B, thus reducing the transcription of type I interferon and the production of pro-inflammatory cytokines [3,4]. The modulation of cytokines has been shown in experimental systems. HCQ inhibits interferon-alpha secretion of plasmacytoid dendritic cells exposed to immune complexes. It also inhibits the release of multiple pro-inflammatory cytokines in vitro. Human evidence is more indirect and is discussed in conjunction with the evidence of biomarkers [3,4,29]. Other more complex targets include autophagy and lysosomal function. HCQ disrupts the process of autophagic flux by inhibiting the degradation of the lysosomes. This action is used in the field of oncology research. It has a molecular mechanism of autophagosome-lysosome fusion blockage [28]. In lupus, there is a net immunological outcome that remains to be completely defined and hence is expressed as a proposed mechanism rather than an established disease-relevant mechanism [4,28]. Other effects on platelets and on metabolism are described. The anti-inflammatory and lysosomal mechanism might be responsible for the improvement of insulin sensitivity and lipid handling by HCQ [14]. It has antithrombotic properties in experimental models, including direct interference with the binding of antiphospholipid-antibody complexes to phospholipid membranes [12,30]. Later these will be discussed as systemic effect mechanisms.

3.1 Molecular mechanisms of hydroxychloroquine and their evidence status

However, the mechanism of action of hydroxychloroquine (HCQ) in systemic lupus erythematosus (SLE) is complex with various interconnected biochemical pathways, though some pathways are supported by more evidence. HCQ is a weak base that accumulates in the acidic intracellular compartments, such as endosomes and lysosomes, where it gets protonated and trapped, a well-known physicochemical property of the drug [3,4]. Eventually, the stored HCQ protonates and buffers the protons in the lumen, enhancing the pH of endosomal/lysosomal compartments and lowering the normal acidity [3,4]. This shift in the pH alters the signal of the Toll-like receptor (TLR) 7 and TLR9, which depends on the proper acidification of endosomes to allow their activation and recognition of nucleic-acid containing ligands. HCQ may also have a direct effect on the nucleic acid and affect its binding to these receptors [3]. TLR7/9 engagement inhibition can therefore lead to decreased downstream signalling via myeloid differentiation primary response 88 (MyD88) and downstream transcription factors, such as interferon regulatory factor 7 (IRF7), nuclear factor kappa B (NF- κ B); this mechanism is mechanistically supported, but the impact of engagement of each downstream component on clinical effects of HCQ is less well established [3,4]. The down-regulation of IRF7-dependent transcription may then result in a diminished production of type I interferons, especially interferon alpha, as seen in experimental models, with some indirect evidence from humans [3,4]. Another potential mechanism of action is the disruption of antigen presentation by increased endosomal/lysosomal pH, which could affect antigen loading onto major histocompatibility complex class II (MHC II) molecules with a reduction in presentation of autoantigen to T lymphocytes, though this is proposed [3,4]. HCQ has also been linked to cytokine regulation, such as decreased interferon alpha, interleukin-6 (IL-6), and tumor necrosis factor (TNF) production in experimental models. Lower levels of certain pro-inflammatory cytokines have been shown in some lupus groups of HCQ users compared to nonusers, but human studies have been inconsistent [3,4,29]. HCQ also disrupts autophagy and lysosomal degradation by impairing the function of the lysosomes and autophagic flux, but the exact immunological effects in SLE are still unclear and how these effects relate to the therapeutic effects of HCQ is not yet known [4]. In addition to its immunomodulatory properties, HCQ has been shown to have antithrombotic effects and antiplatelet activity through experimental and clinical studies, such as inhibitory effects on the activation of platelets and in the interaction of antiphospholipid-antibody complexes with phospholipid membranes [11,12,30]. Last but not least, HCQ could have metabolic effects; observational studies and small clinical trials have found both correlation with improvement in insulin sensitivity and with a modest decrease in circulating cholesterol and triglycerides concentrations [14,15]. Together, these mechanisms suggest that HCQ is known to have effects at several different stages of SLE-associated immune and metabolic dysfunction, from well-defined physicochemical effects to mechanisms that have been supported experimentally or are less well delineated.

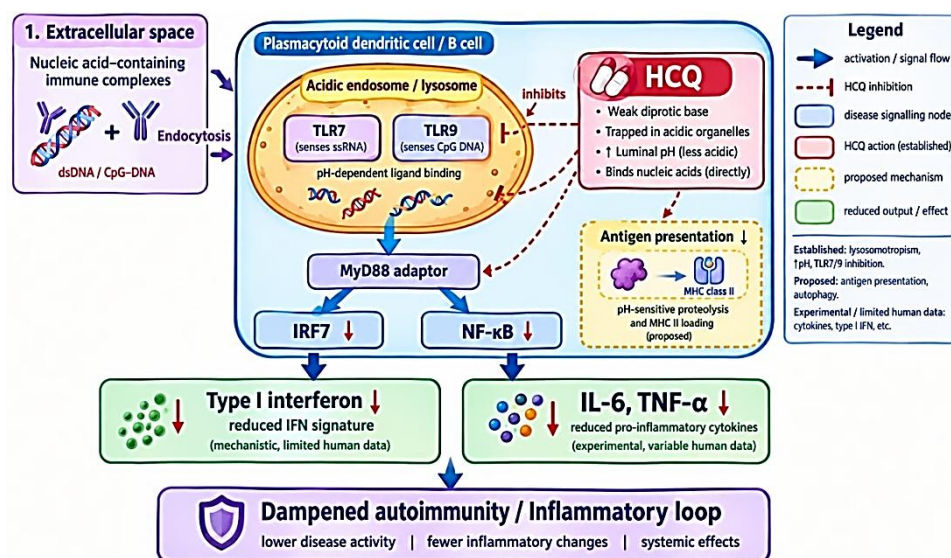


Figure 1: Molecular mechanism of HCQ in SLE [11, 13, 17, 36]

IV. HYDROXYCHLOROQUINE AND CONVENTIONAL DISEASE CONTROL

The best defined clinical use of HCQ is in terms of disease activity control. There was a consistent reduction in activity and fewer flares in a systematic review of antimalarials in SLE [7]. This supports the position statement of the guideline that the majority of patients should be treated with HCQ [2]. Flare prevention is biologically coherent with the mechanism. The interferon loop is damped upstream by HCQ, which should lessen the frequency of immunological escalations. The link is supported by withdrawal studies. Treatment resistance has been linked to increased flares, which

indicates a continuous suppressive activity [7]. Activity control has a biochemical component, encoded in blood concentration. Low HCQ blood levels have been linked to increased disease activity, and to exacerbations [9]. While a randomised trial that titrated doses to optimise blood levels alone did not appear to result in a reduction in flares during the study period, the same trial identified adherence as a significant blood level and outcome determinant [10]. One other result is the need for glucocorticoids. HCQ helps to achieve a good baseline control which can reduce the amount of steroids required to achieve suppression. Guidelines that place HCQ and immunomodulators in the position to 'wean' the glucocorticoids to low or even no maintenance doses [2,6] are replacing these. These are being replaced by guidelines that place HCQ and immunomodulators in the position to 'wean' the glucocorticoids to low or even no maintenance doses [2,6]. Continuation is the preferred treatment option in the long-term. HCQ is well tolerated over many years of use [6,7]. The primary long-term risk associated with it is retinopathy, which is dose and duration dependent and can only be cared for by screening, not by avoidance [19,20]. Activity control meets outcome in cumulative organ damage. Observational cohorts link HCQ to reduced damage accrual and in multiethnic cohorts to better survival [8]. The rationale is that prolonged less activity and less steroid exposure lead to less disease and less steroid damage [6,8]. These affiliations are positive but are still only descriptive.

V. HYDROXYCHLOROQUINE AND LABORATORY BIOMARKERS

For each biomarker the same structure is used. It describes the marker, its significance in SLE, whether HCQ could affect it, its nature of supporting evidence and that it is a validated marker for monitoring. The inherent concern with most human biomarker data for HCQ is noninterventional as opposed to interventional.

5.1 Autoantibodies (ANA, anti-dsDNA, anti-Sm and others)

Anti-double-stranded DNA antibodies are a disease specific, pathogenic response. They increase during active disease, especially during renal disease, and they decrease when the disease is controlled. Anti-dsDNA titres may also decrease indirectly with successful treatment, due to the overall reduction in activity of HCQ [1,7]. Evidence is observational with the added confound of concurrent immunosuppressive therapy. Anti-dsDNA is a good activity marker, but it does not indicate a specific HCQ effect and is only a marker of disease status. Specific, relatively stable reactivities include anti-Smith and other antibodies to ribonucleoproteins. Anti-Sm is highly specific to SLE. During the lifetime of a person, these antibodies remain the same. There is no evidence that HCQ significantly affects them. Their job does not involve response monitoring, it is diagnostic/prognostic [1].

5.2 Complement biomarkers (C3, C4 and related)

Complement proteins C3 and C4 are classical pathway proteins. They are consumed by immune complexes, leaving them in active SLE. If disease control is effective then the consumption will slow and levels can be restored [1,2]. The direct effects of HCQ may play a role in this recovery, by dampening immune-complex mediated activation. The impact is once again indirect and observed. Activity markers, including low C3, C4, particularly with high anti-dsDNA are established and are used in routine monitoring [2]. But they do not demonstrate a drug-specific signal, they demonstrate the disease response. There can be some patients who are genetically deficient in complement, making the situation more complex. Routine laboratories are less standardised in the use of complement split products, which are more dynamic [1].

5.3 Inflammatory biomarkers (CRP, ESR, IL-6, TNF-alpha, interferon)

C-reactive protein is an acute phase protein of the liver, mainly stimulated by Interleukin-6. CRP levels are moderately elevated, if at all, during periods of active SLE; a high CRP should be used as a trigger for checking for infection or serositis. The indirect effect of HCQ on CRP is small. CRP is most useful as a marker for infection, not to track HCQ [1]. The ESR is a measure of plasma protein changes such as fibrinogen and immunoglobulins. Rises and can stay elevated in active SLE. It is nonspecific. No monitoring of HCQ is carried out by ESR, but an improvement in overall activity may result in a drop in ESR [1,7]. Pro-inflammatory cytokines that are downstream of innate activation include interleukins 6 and tumour necrosis factor. These are commonly raised in SLE. Experimental findings indicate that HCQ decreases their production and cohort data indicate that HCQ users have lower cytokine levels [3,4,29]. Human response to HCQ is also unpredictable and there is no routine monitoring assay for HCQ. They are still essentially a research tool [5,29] for their measurement. The most downstream pathway of the HCQ target is the type I interferon pathway. An interferon gene signature is measurable, and there is a corresponding correlation of activity in many patients. The mechanistic action of HCQ is to decrease this signature by inhibiting TLS7/TLS9 signalling [3,4]. To date, there is still limited direct and consistent evidence for the suppression of interferon-signature in humans by HCQ. The interferon signature is a promising but not yet routine biomarker [5,23].

5.4 Haematological and biochemical parameters

The complete blood count is the measure of marrow output, plus peripheral turnover. Through immune mechanisms, SLE may also cause leukopenia, lymphopenia, anaemia and thrombocytopenia. Cytopenias are not a common side effect of HCQ and counts can recover as activity decreases. The blood count is not specifically monitored for HCQ but for disease and other medicines [1,6]. Renal biochemical markers are a reflection of renal glomerular and tubular function. Lupus nephritis affects creatinine, estimated glomerular filtration rate (eGFR), and urinary protein. In the renal section, HCQ is

known to show renal protective effect [17,18]. These markers play a key role in monitoring nephritis. Liver biochemical markers are hepatocellular and cholestatic integrity markers. Raised transaminases may be due to disease, comorbidity or other medications. The incidence of hepatotoxicity due to HCQ is low. Liver tests are routinely performed as a safety check, but they are not a marker of HCQ response [6].

5.5 Potential effects of HCQ on individual laboratory biomarkers

The mechanisms of action of hydroxychloroquine (HCQ) on individual laboratory biomarkers in systemic lupus erythematosus (SLE) are dependent on the biological pathway represented by each biomarker and the strength of the evidence. There is little or no significant change in antinuclear antibodies (ANA) during effective HCQ therapy. ANA is considered to be a relevant diagnostic criterion in SLE, but is not widely used to monitor HCQ therapy, as it has only limited association with treatment response [1,7]. Anti-dsDNA antibody levels may fall during periods of decreased overall disease activity, but may be affected by other immunosuppressive medications that are given at the same time, so anti-dsDNA is likely a measure of disease rather than a measure of the effectiveness of HCQ [1,2,7]. Anti-Sm and anti-RNP antibodies, however, tend to remain stable in the course of treatment and are primarily employed for diagnosis and prognosis and not for routine therapeutic monitoring [1]. Complement components C3 and C4 may rise towards normal levels when the disease activity is controlled, due to decreased immune-complex mediated consumption of complement. Still, complement levels are not specific markers for the response of HCQ therapy, and are markers of disease activity [1,2]. During SLE treatment, there may be only minor or indirect changes of C-reactive protein (CRP) and the usefulness of this assay in evaluating HCQ effectiveness is limited; a significant increase in CRP levels should alert clinicians to the presence of infection or another inflammatory process [1]. Likewise, the erythrocyte sedimentation rate (ESR) can be reduced in response to better disease control, but is not specific and could not therefore be used as a monitoring biomarker for a drug [1]. In experimental systems, HCQ has been shown to reduce inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF), suggesting anti-inflammatory activity, although the results of human studies are conflicting and measuring these cytokines is not widely used outside of research laboratories [3–5]. The type I interferon signature may also decline due to HCQ-mediated inhibition of endosomal nucleic-acid sensing and downstream TLR7/TLR9 signalling, but so far there is only indirect evidence to support this effect specifically in HCQ-treated patients, and so interferon signatures are regarded as emerging rather than routine markers of treatment activity for monitoring therapy [3–5]. Renal biomarkers, such as blood and urine creatinine, estimated glomerular filtration rate (eGFR), and urinary protein level, may help to assess improvement during the treatment of lupus nephritis. They are important parameters to monitor renal involvement, and are supported by evidence from observational and cohort studies, though these do not represent a specific renal disease response, but rather a pharmacological correlation of HCQ. Lastly, blood concentrations of HCQ are a measure of systemic drug exposure and adherence, but do not directly measure SLE activity. Observational studies and clinical trial data indicate that HCQ concentration measurement could be used to help determine sub-optimal exposure, to aid in adherence measurement, and to guide dose selection as a therapeutic-monitoring biomarker; however, the use of this biomarker as a routine tool for therapeutic monitoring is still in development [9–11].

VI. HYDROXYCHLOROQUINE AND RENAL BIOCHEMISTRY

The endpoint of clinical concern for clinical biochemistry laboratories is lupus nephritis. Almost all biochemical and urinary markers define and monitor it. These include serum creatinine, estimated GFR, urinary protein and the urine protein to creatinine ratio [2,17,33]. Excretory kidney function is reflected in serum creatinine and estimated glomerular filtration rate (eGFR). They fluctuate in amount according to the damage to the glomerulus in nephritis. They are late indicators as significant damage can occur before a measurable change can be detected. They still are the mainstay of renal monitoring [2,18]. Glomerular barrier integrity = urinary protein and protein/creatinine ratio. Proteinuria is an early and sensitive active sign of nephritis. The spot protein-to-creatinine ratio can be used as a useful quantitative alternative to the 24-hour collection. It is used to express the outcomes of treatment [2]. The net result is serum albumin. It is within the nephrotic range of disease. Low albumin also has an impact on drug binding and oedema. Active nephritis is a simple and informative biomarker [2]. Certain components of the complement system and anti-double stranded DNA are particularly interesting in renal disease. Renal flares are often accompanied by fall of C3 and C4 levels, and the rise of anti-dsDNA level. This serological pattern is applied in conjunction with urine findings, to determine the activity and to schedule biopsies [1,2]. Renal biomarkers are in development. New urine markers, like monocyte chemoattractant protein-1 and others, are designed to identify activity before creatinine. They do not exist as yet in the standard form. Because they are not confirmed clinical assays they are included here for research purposes. There has been a correlation of HCQ with renal benefit in lupus nephritis. HCQ use was found to be significantly related to reduced renal injury in a multiethnic population [17]. One recent study found that the rate of decline in glomerular filtration rate was slower for HCQ-treated patients with lupus nephritis than for those who were not [18]. The results are consistent and promising. The effect of any renal benefit is likely a complex one. Part is probably indirect, via overall improved disease control and decreased immune-complex nephritis [17]. A direct tissue-protective role is possible, but not established. Importantly, they are observationally derived, and can be confounded by indication and adherence [17,33]. Thus, causality is not proven.

VII. HYDROXYCHLOROQUINE AND METABOLIC BIOCHEMISTRY

The relevance of metabolic biochemistry is that SLE and treatment increases cardiometabolic risk. Dyslipidaemia and insulin resistance are caused by chronic inflammation and glucocorticoids. Laboratory medicine is interested in any drug that neutralizes these effects [14,31]. Modest favourable changes have been observed with HCQ for lipids. The reported results of total cholesterol, low-density-lipoprotein cholesterol and triglycerides are low, and those of high-density-lipoprotein cholesterol vary [14]. The suggested mechanism is a decrease in cholesterol handling by the liver and inflammation. Most of the evidence is observational and from small studies, and thus the effect size is unknown [14]. The signal is more consistent in neighbouring diseases with regard to glucose metabolism. In experimental and clinical studies, HCQ increases insulin sensitivity [14]. A large observational study of rheumatoid arthritis (RA) showed that HCQ was associated with fewer diabetes cases [15]. One possible explanation would be decreased degradation of the internalized insulin in the lysosome and decreased inflammatory insulin resistance [14]. The biomarkers relevant to the disease are fasting glucose, glycated haemoglobin and insulin sensitivity measures. In some patients HCQ decreases fasting glucose and increases sensitivity [14]. HCQ may rarely cause hypoglycaemia, typically when it is used in people with kidney disease and/or an empty stomach. It is a known, although rare, safety point [14]. These metabolic effects link inflammation, metabolism and cardiovascular risk. The proposed mechanism is that, over time, decreased inflammation and glucose and lipid handling would result in decreased atherosclerotic burden [14]. This is a bio-friendly option. It is not exaggeration to say. The metabolic effects are minimal, and mostly observational and therefore do not conclusively demonstrate cardiovascular risk reduction [14,16].

VIII. HYDROXYCHLOROQUINE, CARDIOVASCULAR BIOLOGY, AND THROMBOSIS

Patients with SLE have an increased risk of cardiovascular and thrombotic disease. Several drivers combine. Inflammation damages the endothelial cells. Metabolic risk is added by dyslipidaemia and glucocorticoids. APLs are a type of antibody that encourage blood clotting. This leads to accelerated atherosclerosis and increased thrombosis rate in comparison with general population [1,14,31]. HCQ may have anti-inflammatory effects in the endothelium. Suppression of interferon and cytokine tone may lead to better endothelial function [3,4]. This mechanism is feasible, but has been indirectly supported, with limited direct human endothelial-function data available for HCQ. HCQ, in models, has measurable anti-thrombotic properties on platelets and coagulation. May be antiplatelet and will break down platelet activation and aggregation. It also directly inhibits the binding of antiphospholipid-antibody complexes to phospholipid bilayers [12,30]. These are the mechanisms of reduced clotting tendency.

Observational clinical thrombosis data are supportive. HCQ has been suggested as an antithrombotic agent in APS as it possesses antithrombotic properties in its biology [12]. A cohort study [11] in SLE found that increasing blood levels of HCQ were associated with a decreased risk of thrombosis. This is an association with a biochemical exposure marker and a hard vascular outcome [11]. In the context of lipids and systemic inflammation, the prior metabolic effects are pertinent to vascular risk. A modest improvement in lipid profile and reduction in inflammation might delay atherosclerosis [14]. Again, the data are indirect. There is no evidence from a randomised trial that HCQ prevents myocardial infarction or stroke in SLE. Two points should be noted about antiphospholipid antibodies and antiphospholipid syndrome: These antibodies have a high prevalence in SLE and increase the likelihood of obstetric and thrombotic problems. At the membrane level, HCQ seems to be opposite in action to some of their prothrombotic effects [12,30]. This has led to the idea that HCQ may be an attractive add-on in some ANA-positive patients, but anticoagulation therapy is the foundational therapy for those with established syndrome [12]. The conclusion is one of biological plausibility but not proof. Antithrombotic and anti-inflammatory mechanisms are connected. Blood-level and thrombosis association are suggestive of this, and are encouraged by observations associations [11,12]. Randomised data on demonstrated reduction in cardiovascular events is not yet available. Plausibility should not be given as proven clinical benefit.

IX. HYDROXYCHLOROQUINE AND GLUCOCORTICOID-SPARING EFFECTS

The theme of SLE treatment is minimizing the use of glucocorticoids. Steroids have an immediate effect on activity but can have an effect that builds over time. The suggested chain is simple. The use of HCQ leads to better disease control and fewer flares. With better control, less steroid dose will have to be used. If exposure to steroids is reduced, then so are the effects of steroids [2,6,25]. The first priority is the metabolic problems of steroids. Glucocorticoids increase blood sugars and may trigger diabetes. They worsen dyslipidaemia. Some of this metabolic damage can be prevented if HCQ enables the body's nutritional demands to be met with lower amounts of steroids. There may also be a direct beneficial metabolic effect of HCQ, discussed above [14,15]. Hypertension and cardiovascular consequences are the next target. Steroids increase blood pressure and help cause atherosclerosis. This will be eased by a reduction in steroid exposure. It is worth noting that if HCQ provides any further direct vascular benefit it would be extra [6,16]. There are also skeletal and infective complications. Steroids decrease bone density and increase susceptibility to infection. Both are expected to decrease with the effective

background therapy, which is steroid-sparing. However, it has no effect on increasing infection risk like immunosuppressive drugs, which is a positive feature in this regard [6,7]. The question of the interpretative challenge is attribution. It can be difficult to determine the effects of HCQ over and above that of decreased steroid use, when a patient responds well. The interferon-pathway action is likely to be direct, among the other benefits [3,4]. Other indirect effects are due to decreased steroid exposure, for example, steroid-induced diabetes [6]. Steroid dose should be modelled as a mediator, rather than a confounder in careful studies.

X. HYDROXYCHLOROQUINE AND LONG-TERM ORGAN AND SYSTEMIC OUTCOMES

The cumulative benefits of HCQ are the long-term benefits. These are damage accrual, renal preservation, cardiovascular and thrombotic events, hospitalisation and survival. There are more observational cohorts as evidence, and thus these should be interpreted critical [5,8]. Cumulative organ damage is a validated long term measure in SLE. Damage accrual has been associated with less damage when using HCQ across cohorts [6,8]. The mechanism is believed to be attributed to diminished activity and steroid exposure. Association does not equate to causation [8]. Discussion about renal outcomes has been discussed. In patients with lupus nephritis, HCQ has been linked to less renal damage and slower renal filtration decline [17,18]. The potential benefits on cardiovascular and thrombotic outcomes are plausible but not proven, with a strong signal in the case of the blood level association and the thrombosis association [11]. The most commonly quoted outcome in the HCQ's favour is survival. In a multiethnic cohort of LUMINA, there was an association between HCQ use and survival [8]. This discovery has proven to be significant. However, it is constrained by the same limitations of observation, such as the patients who are healthier or more adherent are more likely to take and adhere to HCQ [8]. People who are sick are likely to be hospitalised and develop complications from the disease, which tends to go along with activity. Reducing those two should be possible with better control. This is consistent with cohort data which show a reduction in severe events during HCQ therapy [5,7]. The direction is good and the causal certainty is restricted as in all countries. To be honest, HCQ is linked with improvements in long-term outcomes in several areas, but there are no randomised long-term outcome trials. Ethical justification to withhold a guideline-recommended, low-toxicity drug for conducting a placebo-controlled survival trial may be more difficult today. This is reason enough this evidence is mostly observational [2,7].

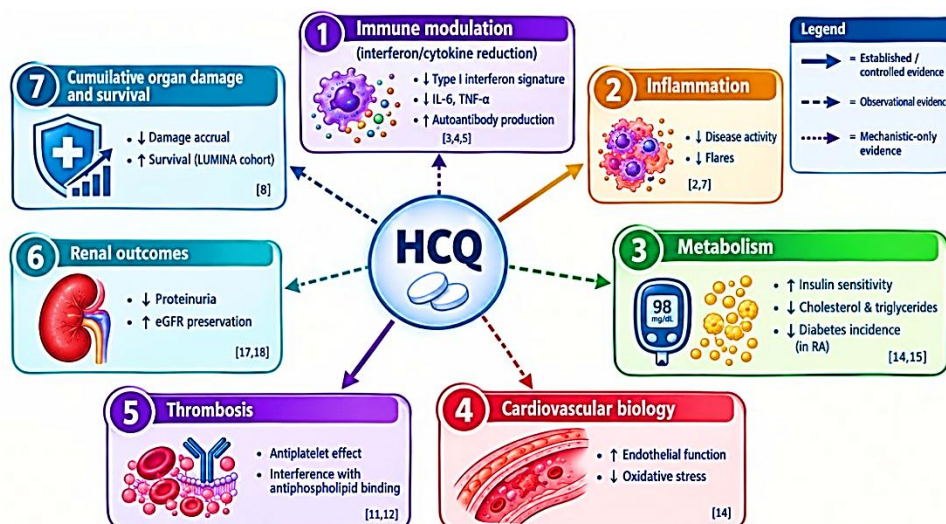


Figure 2: Beyond disease control: HCQ across systems [11, 12, 15, 36]

In addition to its proven ability to impact disease activity, there are a number of systemic benefits of hydroxychloroquine (HCQ) in systemic lupus erythematosus (SLE) patients, but the evidence supporting these benefits is variable and causality is uncertain. Use of HCQ has been linked to survival, and in multiethnic cohort LUMINA, use of HCQ was associated with better survival; however, due to the nature of the evidence, from a large observational cohort, it is not possible to conclude that treatment with HCQ is the cause of improved survival [8]. Likewise, there have been reports of decreased damage accrual in multiple cohorts, and this association cannot be extrapolated to confirm causality due to the observational design of the studies [6,8]. The evidence for a protective effect of HCQ against renal damage and for a slower decline in GFR in lupus nephritis is predominantly from cohort studies, so there is a degree of association, but no evidence of a definite causal renal-protective effect [17,18]. Concerning thrombosis, there are cohort data showing that increasing blood levels of HCQ are associated with a decreased risk of thrombotic events, but it should also be noted that there is an

association and not a causal relationship [11]. Moreover, there is biological plausibility for HCQ to have an antithrombotic effect because of mechanistic and observational evidence that it can inhibit antiphospholipid antibody prothrombotic effects [12]. In the metabolic domain, there has been an association of lower diabetes incidence in persons with RA who take HCQ, although this is supported in large observational data; this does not imply a causal preventive effect [15]. At last, in the pregnant state, there is limited, but supportive, open label evidence that HCQ use is associated with lower rates of recurrence of congenital heart block in pregnant mothers at risk [13]. Overall, these results indicate that the potential effects of HCQ are not only limited to suppression of SLE activity but that they also hold promise for other effects, though the evidence supporting these findings is not consistent, and much is from observational studies, which can be confounded and otherwise limited.

XI. HYDROXYCHLOROQUINE IN PREGNANCY AND SPECIAL CLINICAL POPULATIONS

SLE is a high-stakes pregnancy. Both mother and fetus are at risk of developing active disease. Note: HCQ is thought to be safe to continue during pregnancy, since there is a risk of a flare-up if it is stopped [2,13,26]. This is one of the very few diseases directed drugs that is still continued during pregnancy. The first benefit is maternal disease control. In lupus pregnancy, patients who have continued HCQ show decreased disease activity and fewer flares [2,32]. Control enhances the safety of the mother and her unborn child and may decrease the use of more dangerous medications or high doses of steroids while the child is in the womb [2,6]. Fetal and neonatal outcomes are the second consideration. The most specific evidence relates to anti-Ro/SSA-positive pregnancies. Such antibodies may lead to congenital heart block in the fetus. A prospective, open-label trial found that maternal HCQ was linked to a decreased risk of anti-SSA/Ro-associated CHB [13]. This is an important obstetric advantage, consistent with its mechanisms. Counselling is thus directed by anti-Ro/SSA and anti-La/SSB antibodies. HCQ prophylaxis is most recommended for women with these antibodies and a previously affected pregnancy [13]. These pregnancies continue to receive fetal cardiac surveillance. Dosing and safety considerations include special population: renal impairment. HCQ is excreted partly in the urine. Exposure is increased and the risk of retinopathy and, rarely, hypoglycemia [19,20] increased with impaired function. Dose and screening should be tailored to this [19]. Individual treatment is required to those at higher risk for toxicity. These include individuals with a risk for retinal disease or maculopathy, a very long duration of use, a high weight-based dose, and those with concurrent tamoxifen therapy or renal dysfunction [19,20]. In these patients, especially, the benefit-risk balance and level of monitoring should be discussed.

XII. SAFETY, TOXICITY, AND LABORATORY OR CLINICAL MONITORING

It's a central issue of HCQ's relative safety. It is well tolerated by most patients for years [6,7]. The monitoring requirements are thus concentrated on a few significant and mostly dose and duration-related toxicities [19,20]. The most important long term risk is retinal toxicity. An unusual maculopathy has been associated with HCQ, which may continue after the drug has been discontinued. There is a cumulative dose and duration relationship for prevalence. However, in a large study, overall toxicity was about 7.5% among long-term users and increased significantly with the duration of use, particularly at the higher weight based dose [20]. Renal impairment, and concurrent tamoxifen use, are risk factors, and, at doses > 5 mg/kg, the risk of retinopathy for a recent cohort was estimated at about 8.6% at 15 years [20,34]. The two modifiable factors are dose and cumulative exposure. Recommendations for current ophthalmology guidelines include a daily dose of approximately 5 mg per kg (real) body weight and screening accordingly [19,34]. Baseline screening and periodic monitoring (usually by automated visual field, optical coherence tomography) enable early detection before loss of central vision occurs [19]. Exposure and renal function interact. Impaired kidney function increases the risk of toxicity and blood levels [19] due to its contributing role to clearance. Therefore, renal status must be taken into account when deciding the dose and frequency of screening [19,20]. Cardiac toxicity is rare, but significant. HCQ has the potential to cause QT prolongation, especially in combination with other drugs that prolong the QT interval or electrolyte abnormalities. Rarely, chronic use causes a cardiomyopathy, sometimes with conduction disease [16]. A systematic review was reported documenting these cardiac complications and their dose/duration relationship [16]. Neuromuscular toxicity occurs only very rarely. Long-term use can cause a vacuolar myopathy, and sometimes a neuropathy as well. Practical monitors of clinical vigilance and, if indicated, creatine kinase and examination [16]. Drug interactions, such as additive QT effects, and drug level interactions, should be checked at every prescription [16]. The common thread is that monitoring turns a potentially hazardous toxicity profile into a manageable one. Correct dosing and screening are largely effective in prevention of retinopathy at a population level [19,20]. Rare and detectable cardiac and neuromuscular events occur. Realising the therapeutic benefit is only possible safely over decades through appropriate monitoring [16,19].

12.1 HCQ toxicity and recommended monitoring

Hydroxychloroquine (HCQ) should be monitored for several organ systems, especially if the treatment is to be continued for long periods of time, due to some side effects being dose- or duration-dependent and related to drug clearance. The most important long-term toxicities include retinopathy and maculopathy, which are more likely with high cumulative

dose and longer treatment times. The risk is also increased when using a daily dose > about 5 mg/kg of real body weight, renal impairment and concurrent tamoxifen therapy. Monitoring of the eye involves a baseline exam and then automated visual-field testing and spectral-domain optical coherence tomography (SD-OCT); standard annual monitoring starts after about five years of treatment; and is recommended in patients with other risk factors [19,20,34]. A cardiac safety issue is QT prolongation, especially if HCQ is used in conjunction with other QT-prolonging drugs or when there are electrolyte abnormalities. Therefore, consideration of electrocardiography and serum electrolytes should be made when prescribed with other drugs that can prolong the QT interval should be considered, in patients with the appropriate risk factors. [16]

Rare complications include cardiomyopathy and conduction abnormalities, which have been mostly linked to prolonged and cumulative HCQ exposure. If these complications are clinically suspected, clinical assessment, electrocardiography and echocardiography may be performed [16]. Another rare chronic toxicity is neuromyopathy which can present as vacuolar myopathy and less commonly peripheral neuropathy. When muscle weakness (or other neuromuscular symptoms) occurs, clinical assessment is indicated and a blood test of creatine kinase (CK) may assist in the evaluation of suspected myopathy [16]. Hypoglycaemia is rare, but may be caused by a greater effect of HCQ on insulin, and can occur in situations of renal impairment, or in fasting. Therefore, blood glucose should be measured when appropriate signs and symptoms are present [14]. Finally, drug exposure in renal impairment is a consideration, because of the lower renal clearance that can lead to higher HCQ level and thereby higher risk of toxicity. Renal function should be monitored during therapy, and renal impairment significantly may require dose adjustment with more frequent monitoring of toxicity; in selected patients, measuring the blood concentration of HCQ may be helpful [19].

XIII. HYDROXYCHLOROQUINE BLOOD CONCENTRATION AND THERAPEUTIC DRUG MONITORING

Pharmacokinetics of HCQ is complex. Oral bioavailability is high with some variation. The drug is widely distributed in blood cells and tissues. It has a long half-life at the end of its useful life, weeks. A steady state was obtained over a relatively long period that is similar to the slow onset of clinical effect [3,6]. There is significant inter-individual variation. The plasma levels achieved by the same dose are quite variable between patients. All of these aspects are contributing. This diversity is the reason why blood concentrations are measured, and not exposures from dose alone [9,10]. The blood concentration is a good marker of adherence. If it's very low or not present at all, it's a very good indicator of non-adherence, not fast metabolism [9,10]. Non-adherence has been reported to occur in SLE and is a trigger of flares, making an objective measure of adherence useful clinically [10,35]. The link between concentration and response is supported but is subtle. Low HCQ levels has been linked with higher disease activity and exacerbation [9]. However, a randomised trial in which doses were increased in order to increase blood levels did not show this to be the case, and dampened enthusiasm for simple level targeting [10]. There is now the safest current evidence which suggests that a target is not essential to reach, but an adequate exposure is [9,10]. The dependence of concentration-toxicity is beginning to be understood as well. Very high exposure is a likely risk factor for retinopathy, in line with the dose/duration data [19,20,34]. The positive side, however, was that higher exposure was for individuals in a cohort correlated with reduced risk of thrombosis [11]. So the personalised medicine application is a promising but constrained one. Proper levels of HCQ can be used to verify compliance, understand non-response and identify excessive exposures [9,10,11]. Today best viewed as a commitment and precautionary measure. However, routine dose titration, to a targeted efficacy level, is still an emerging application that has not been established [10, 11].

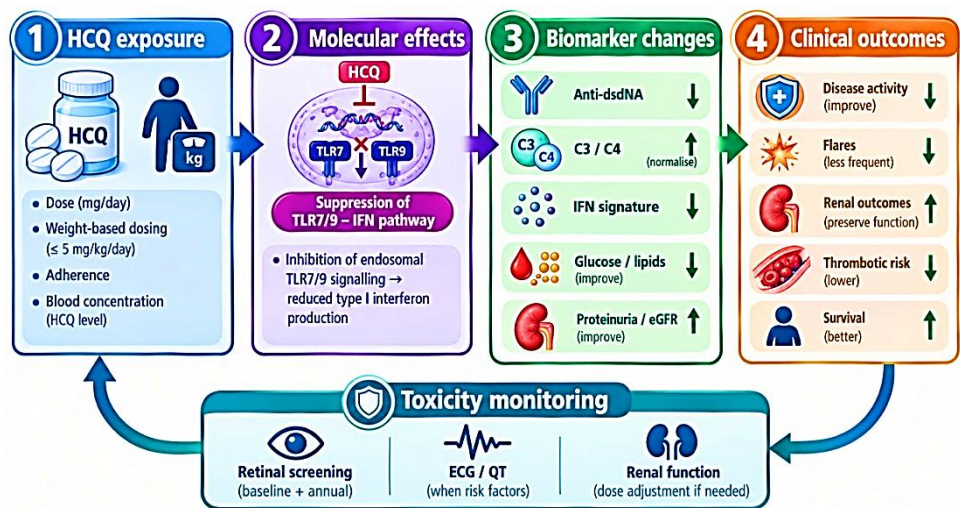


Figure 3: Clinical biochemistry framework for HCQ therapy [3, 9, 19, 36]

XIV. BENEFITS, MECHANISMS, BIOMARKERS, AND OUTCOMES LINKED TO HCQ

The potential clinical effects of hydroxychloroquine (HCQ) in systemic lupus erythematosus (SLE) can be associated with specific biological effects, laboratory markers, and clinical outcomes, which exist with varying degrees of evidence. Although increased activity of the TLR7/TLR9–type I interferon pathway is the primary driver of lower disease activity, other immunological measures such as changes in anti-double-stranded DNA (anti-dsDNA) antibodies, complement C3 and C4 levels, and the type I interferon signature have been recognized as indicators of immunological activity and may also be responsible for lower disease activity, as seen with HCQ therapy [2,7]. The steroid-sparing effects could be due to better background regulation of SLE activity, allowing a reduction of exposure to glucocorticoids. This can be measured by monitoring glucocorticoid dose and validated disease-activity indices and a decrease in cumulative steroid exposure can then reduce steroid-related toxicity [6]. Renal protection might be related to downregulated immune complex-mediated renal inflammation, especially in the context of lupus nephritis. Biomarkers that are relevant include urinary protein excretion, estimated glomerular filtration rate (eGFR), and complement levels; observational studies have suggested that HCQ might slow the rate of renal function decline [17,18]. It has been suggested to have an antithrombotic effect by inhibiting platelet activation and blocking the binding of antiphospholipid antibodies to phospholipid membranes. The levels of HCQ in the blood and whether or not it is an APA may be useful in this pathway, and being lower is associated with a lower risk of thrombosis has been reported in observational studies [11,12]. There may also be a metabolic benefit, perhaps with regard to insulin sensitivity, and limited effects on lipids. These metabolic changes can be evaluated with fasting glucose, glycated haemoglobin (HbA1c) and lipid concentrations and observational evidence has linked HCQ use with decreased incidence of diabetes, especially in patients with rheumatoid arthritis [14,15]. During pregnancy, this has been suggested as a benefit of the obstetrical system because of the possibility of a decrease in the effect of maternal anti-Ro/SSA antibodies. Anti-Ro/SSA antibody status is used to identify pregnancies with increased risk of recurrence; and prospective studies have confirmed an association between HCQ use and lower rates of recurrence of congenital heart block [13]. Lastly, a potential survival benefit may relate to the cumulative benefit of improved disease control, less organ damage and decreased exposure to glucocorticoids. Observational studies have shown improved survival of HCQ users, even though this has not been proven to be causal; the cumulative damage index can be used to monitor long-term disease consequences [8].

XV. EVIDENCE GAPS AND CONTROVERSIES

Systemic evidence is primarily observational for HCQ. This gives rise to identified specific weaknesses. They need to be communicated in a way that will not result in the claiming of benefits that are not due [5,8]. The main issue is confounding by indication. HCQ is used in specific patients at the discretion of clinician. Such patients might have certain systematic differences from non-users that relate to outcome. Thus, some of the benefits observed may be confounded by the population that has access to the drug [8,17]. Add to this treatment-selection and adherence biases. Patients who take and persist with HCQ are more adherent and health engaged. Adherence is a predictor of outcomes, independent of any drug benefit. This can lead to over-estimation of apparent benefit and a study of blood-levels reveals the magnitude of the adherence problem [10]. Synthesis is constrained by methodological heterogeneity. There are a variety of variations in the dose and duration of HCQ treatment, depending on the study. There are differences in the severity of the disease and population composition. They are different in the laboratory assays themselves for the same biomarker which means that a value reported from one laboratory may be different to another [5,10]. It is not easy to pool across these differences. It is difficult, therefore, to conclude that any systematic effects are caused by these. There are controlled data supporting disease control and flare reduction [7]. The survival and advantages in terms of damage, renal and cardiovascular benefits are largely based on associations [8,17]. The interferon-pathway mechanism is coherent and has some indirect human confirmation for HCQ, although less [3,5]. There is still a lack of standardized biomarkers. No standard, routinely-used interferon-signature assay exists. No consensus of HCQ blood level reference range or efficacy target has been established [5,10]. The systemic effects, especially metabolic and cardiovascular, are still unclear as to their magnitude and clinical significance [14,16]. These gaps are what is currently known, but only with honesty.

XVI. CONCLUSION

Hydroxychloroquine is a well-established, safe drug for use in SLE. It decreases disease activity, decreases flares and is recommended for most patients. Its value is now recognized to go beyond just suppressing clinical activity. It has several biological effects. These include biochemical processes in the lysosomes, innate nucleic-acid sensing, interferon and cytokine signaling, and, more tentatively, metabolic and vascular biology. This width is the reason why HCQ is an interesting molecular-medicine molecule. Clinical biochemistry gets you from the mechanism to practice. Measurable outputs of molecular action: autoantibodies, complement, renal and metabolic panels, emerging interferon signature, HCQ blood levels. The measurements help to diagnose, monitor, safety and, more and more, adherence. A balance should be achieved. Proposed systemic effects such as survival, renal effects, metabolic effects and cardiovascular effects are not well established. They

rely primarily on data from observations and need more evidence in the future. Reporting plausibility should not be considered as proof. The future probably will be a tailored drug that's used in a lab environment. Correct dosing, adherence testing, biomarker development and toxicity screening will be further integrated. In this context HCQ represents a good illustration of the reuse of a well-known therapy without over-optimism on the part of clinical biochemistry.

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