THE EFFECT OF SHORT-TERM HYDROXYCHLOROQUINE USE ON CRITERIA AND SELECTED NON-CRITERIA ANTIPHOSPHOLIPID ANTIBODY TESTS

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ABSTRACT

Background/Purpose: One proposed mechanism of antiphospholipid antibody (aPL)-mediated thrombosis is disruption of the Annexin A5 (AnxA5) anticoagulant shield, allowing initiation of coagulation reactions on phospholipid surfaces. The AnxA5 resistance assay (AnxA5-RA) measures resistance to AnxA5 anticoagulant activity in the plasmas of aPL-positive patients. Based on *in vitro* studies, hydroxychloroquine (HCQ) interferes with aPL binding to cell surfaces and helps repair disrupted AnxA5 shields*;* however, no *in vivo* human studies exist. Other non-criteria aPL tests, including anti-domain-I β_2-glycoprotein-I IgG (aDI-β_2GPI) and activated protein C (APC) resistance may be associated with thrombosis in patients with antiphospholipid syndrome (APS). The purpose of this prospective study is to determine the effect of HCQ on the AnxA5-RA, as well as criteria and other non-criteria aPL tests, in persons with aPL.

Methods: We recruited persons with aPL (lupus anticoagulant [LAC], anticardiolipin antibody [aCL] $\geq$ 40 GPL/MPL, and/or anti-β_2-glycoprotein-I [aβ_2GPI] antibody $\geq$ 40 SGU/SMU at two time points at least 12 weeks apart) starting HCQ to give blood at baseline, 6 weeks, and 12 weeks (primary outcome measure: AnxA5-RA; secondary outcome measures: LAC, aCL, aβ_2GPI, aDI-β_2GPI, APC resistance, and D-dimer). As a control group, we also recruited aPL (LAC, aCL, and aβ_2GPI)-negative systemic lupus erythematosus (SLE) patients starting HCQ . We compared the baseline

characteristics of patients with aPL to aPL-negative SLE patients (Mann-Whitney test) as well as a change in results from baseline to week 12 (Wilcoxon signed-rank test).

Results: We compared baseline data from 21 aPL-positive patients (mean age 44.7 ± 11.0 years [range 27-61], 15 [71%] female, 17 [81%] Caucasian; 13 had APS and 8 had asymptomatic aPL) to 12 aPL-negative SLE patients (mean age 45.4 ± 15.0 years [range 22-64], 12 [100%] female, 7 [58%] Caucasian). Mean baseline values for AnxA5-RA were lower (more abnormal) for aPL-positive subjects compared to aPL-negative subjects (134.1 ± 15.6 vs. 168.3 ± 21.9; $p<0.0001$); no patients in the aPL-negative group exhibited resistance to AnxA5 anticoagulant activity. Mean baseline values of aDI-β_2GPI were higher for aPL-positive subjects as compared to aPL-negative subjects (4.40 ± 4.38 vs. 0.76 ± 0.44; $p=0.0002$); no patients in the aPL-negative group had aDI-β_2GPI. There was no significant change between mean baseline and week 12 values for LAC, aCL, aβ_2GPI, AnxA5-RA, aDI-β_2GPI, APC resistance, or D-dimer in aPL-positive subjects receiving HCQ (Table).

Table: Comparison of Baseline and Week 12 Values for Non-criteria aPL Tests in aPL-positive subjects (n=17)

	Baseline	**12 weeks**	**p**	**Reference Range**
AnxA5-RA (%) (Mean ± SD)	136.6.1 ± 16.0	143.96 ± 25.6	0.2435	Negative ≥153 Borderline 140-152.9 Positive <140
aDI-β_2GPI (mg/L) (Mean ± SD)	4.45 ± 4.80	4.01 ± 4.40	0.9265	Negative ≤1.66 Borderline 1.66-2.29 Positive >2.29
APC resistance ratio*	2.69 ± 0.63	2.52 ± 0.92	0.7002	Abnormal <2.0
D-dimer (µg/mL) (Mean ± SD)	1.73 ± 0.72	1.58 ± 0.72	0.1743	Normal 0.8-2.3

AnxA5-RA: Annexin-A5 resistance assay; aDI-β_2GPI: anti-domain-I β_2-glycoprotein-I antibody; APC: activated protein C; SD: standard deviation

* n = 14

Conclusion: Patients with aPL have positive AnxA5-RA and aDI-β_2GPI; our findings support the use of these non-criteria tests to detect aPL. HCQ use was not associated with a change in AnxA5 anticoagulant activity or other criteria or non-criteria aPL tests; duration of HCQ treatment, HCQ dosing, sample size, and lack of efficacy *in vivo* are possible explanations for these findings. Our findings suggest that HCQ may not act through an AnxA5 resistance mechanism.

BIOGRAPHICAL SKETCH

Alana B. Levine, M.D. is an Assistant Attending Rheumatologist at Hospital for Special Surgery and an Assistant Professor of Medicine at Weill Cornell Medicine in New York City. She specializes in the care of patients with autoimmune rheumatic diseases including systemic lupus erythematosus, antiphospholipid syndrome, undifferentiated connective tissue disease, and rheumatoid arthritis and has a particular interest in pregnancy and rheumatic disease. During her fellowship, Dr. Levine cared for complex patients with lupus and antiphospholipid antibodies and her research focused on novel treatments for these patients.

Dr. Levine received her bachelor of arts from Williams College and her medical degree from New York University School of Medicine. She completed her residency in Internal Medicine at New York Presbyterian Hospital–Weill Cornell Medical Center and her Rheumatology Fellowship at Hospital for Special Surgery.

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Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder of thrombosis and/or pregnancy complications combined with antiphospholipid antibodies (aPL), classified by the Sapporo criteria,[1] which require laboratory tests to be positive at two points in time, at least 12 weeks apart, and must include at least one of the following tests: lupus anticoagulant (LAC), anticardiolipin (aCL) enzyme-linked immunosorbent assay (ELISA), and/or anti-β_2-glycoprotein-I (aβ_2GPI) ELISA.

A proposed mechanism for thrombosis in APS is aPL-mediated disruption of the annexin A5 (AnxA5) layer that shields phospholipid bilayers from aPL binding. AnxA5 is a potent anticoagulant protein on the surfaces of platelets and endothelial cells that protects against thrombosis *in vitro*.[2] Plasma containing antiphospholipid antibodies may disrupt the anticoagulant shield produced by AnxA5; clotting may ensue. The AnxA5 resistance assay (AnxA5-RA) is a functional coagulation assay that defines resistance to AnxA5 anticoagulant activity; it measures the degree to which antiphospholipid antibodies in the patient plasma interfere with the protective AnxA5 layer.[3]

Antiphospholipid antibodies against the phospholipid binding protein, β_2GPI, are closely associated with thrombosis in APS. β_2GPI comprises 5 domains (DI-V); aβ_2GPI targeted against the N-terminal domain of β_2GPI (known as domain I), are anti-domain-I β_2GPI antibodies (aDI-β_2GPI) and are strongly associated with both obstetric and thrombotic events. In addition, aDI-β_2GPI activity correlates with AnxA5 resistance in LAC-positive samples, suggesting a link between aDI- β_2GPI and LAC.[4]

Acquired resistance to activated protein C (APC) occurs in APS patients and is a proposed mechanism for thrombosis[5]; APC resistance may function as a non-criterion aPL test in patients who do not possess the Factor V Leiden mutation. Elevated levels of D-dimer, a nonspecific clinical marker of thrombosis, correlate with arterial and venous thrombosis.[6,7] .

Hydroxychloroquine (HCQ), an antimalarial drug widely used in the treatment of autoimmune disorders like systemic lupus erythematosus (SLE), has anticoagulant properties. In aPL-injected mice, HCQ decreases the thrombus size and the time of thrombus formation in a dose-dependent manner.[8] Rand et al. demonstrated that HCQ reverses the binding of aPL-ß$_2$GPI complexes to phospholipid bilayers and protects the AnxA5 anticoagulant shield from disruption by aPL *in vitro*.[9,10] Studies of large lupus cohorts demonstrate that HCQ decreases risk of thrombosis.[11-14] Of note, despite the lack of high quality clinical evidence, clinicians prescribe HCQ "off-label" for the management of persons with aPL without other systemic autoimmune diseases, especially for those with non-criteria manifestations of aPL.

Here we evaluate the effect of short-term HCQ use on both criteria (LAC, aCL, and aβ_2GPI) and non-criteria (AnxA5-RA, aDI-β_2GPI, and APC resistance) aPL tests in persistently aPL-positive individuals.

Materials and Methods

Study Design

We conducted a single-center, observational, hypothesis-generating pilot study in which patients with aPL subjects starting treatment with HCQ as part of standard of care were followed over a 12-week period. The primary objective was to explore the effect of HCQ on AnxA5 resistance. The secondary objectives were to explore the effects of HCQ on LAC, aCL, aβ_2GPI, aDI-β_2GPI APC resistance, and D-dimer.

Study Population

The target population included aPL-positive individuals (both those with APS and those with aPL but without vascular thrombosis or pregnancy morbidity), with or without SLE. We recruited subjects from private rheumatologists' offices and the Lupus Clinic at the Mary Kirkland Center for Lupus Care at Hospital for Special Surgery (HSS).

Inclusion criteria

Eligible persons were age 18 to 65 who had received a prescription for HCQ and who fulfilled at least one of the following criteria: 1) a positive LAC test, as defined by the International Society on Thrombosis and Haemostasis,[8] on 2 occasions, at least 12 weeks apart, 2) a positive aCL IgG and/or IgM isotype (at least 40 units) on 2 occasions, at least 12 weeks apart, and/or 3) a positive aβ_2GPI IgG and/or IgM isotype (at least 40 units) on 2 occasions, at least 12 weeks apart.

We recruited individuals with SLE (fulfilling at least four of the criteria for the American College of Rheumatology for the classification of SLE[9]) into two aPL-

negative control groups – those starting HCQ and those not starting HCQ. Negative aPL was defined by all three of the following within one year prior to enrollment: 1) a negative LAC test, 2) aCL IgG/IgM/IgA isotype < 20 units, and 3) aβ_2GPI IgG/IgM/IgA isotype < 20 units.

Exclusion criteria

Patients were ineligible if they fulfilled any of the following criteria : HCQ or chloroquine use within the past 6 months; prednisone or other steroid equivalent at a dose of 0.5 mg/kg at the time of enrollment; current heparin use; the use of any disease-modifying anti-rheumatic drug, cytotoxic, or biologic medication in the past 3 months; pregnancy; acute thrombotic event in the past 2 weeks; or active malignancy.

Study interventions

The Institutional Review Board at HSS approved the study design, which complied with the Health Insurance Portability and Accountability Act. All subjects gave written informed consent. The study is listed on ClinicalTrials.gov (NCT01475149).

Baseline data collection included demographic information, SLE- and aPL-specific histories, historical aPL profiles, medications, and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)[10] and Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index (SDI)[11] measurements. We conducted follow-up visits at weeks 6 and 12 and interviewed study participants by telephone using a standardized questionnaire at weeks 1, 3, and 9 to assess for compliance with HCQ and side effects.

We collected serum and plasma samples at baseline, 6, and 12 week visits for freezing and storing for later analysis by the clinical laboratory at Montefiore Medical Center. We tested samples for LAC; aCL IgG, IgM and IgA; aβ_2GPI IgG, IgM and IgA; aDI-β_2GPI antibodies; APC resistance; and D-dimer assay.

Lupus anticoagulant was measured using the STA®-Staclot® DRVV Screen and STA®-Staclot® DRVV Confirm lupus anticoagulant kits (Diagnostica Stago, Parsippany, NJ), according to the manufacturer's instructions. Anticardiolipin IgG, IgM, and IgA and aβ_2GPI IgG, IgM, and IgA were measured using Anti-Cardiolipin EIA and Anti-β_2 Glycoprotein I EIA test kits (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Annexin A5 resistance and aDI-β_2GPI IgG were measured as previously described.[12] Activated protein C resistance was measured using a Chromogenix APC Resistance-V kit (DiaPharma Group, Inc., West Chester, OH) in accordance with the manufacturer's instructions. D-dimers were measured using Innovance D-dimer kits (Dade Behring, Marburg, Germany), according to the manufacturer's instructions.

Statistical analysis

To compare baseline medians between the different groups, we used the Mann-Whitney test. To compare median baseline and week 12 values within groups, we used the Wilcoxon signed-rank test (paired differences).

Results

We screened one hundred thirty-three patients, of whom we recruited thirty-three between November 2010 and January 2013. Patients with aPL starting HCQ constituted two groups, those with APS and those with asymptomatic aPL. We recruited control subjects with SLE only (aPL-negative) into one of two groups, those starting HCQ and those not starting HCQ.

Table 1 shows the baseline demographic and clinical characteristics of participants in the study. The majority of the population was female (82%) and Caucasian (73%). All APS patients had thrombotic events in the past; none had a history of obstetric events.

Table 1. Baseline demographic and clinical characteristics of study participants

	aPL positive		aPL negative	
Characteristic	APS (n=13)	Asymptomatic aPL (n=8)	SLE only starting HCQ (n=8)	SLE only not starting HCQ (n=4)
Age, mean ± SD years	44.2 ±10.9	45.4 ±11.7	39.7 ±15.1	56.8 ±6.3
Female	8 (61.5)	7 (87.5)	8 (100)	4 (100)
Race				
Asian	1 (7.7)	1 (12.5)	0	0
African American	1 (7.7)	0	2 (25)	1 (25)
Caucasian	10 (76.9)	7 (87.5)	5 (62.5)	2 (50)
Other	0	0	1 (12.5)	1 (25)
Pacific Islander	1 (7.7)	0	0	0
Hispanic	3 (23.1)	1 (12.5)	3 (37.5)	0
aPL status				
+LAC	8 (62)	4 (50)	0	0
+aCL	8 (62)	4 (50)	0	0
+aβ_2GPI	11 (85)	8 (100)	0	0
Mean SLEDAI at enrollment	1.15	1.375	2.25	5
Mean SLICC at enrollment	1	0.625	1.125	4.75
SLE	2 (15)	2 (25)	8 (100)	4 (100)
Medications				
Aspirin	6 (46.2)	5 (62.5)	0	1 (25)
Warfarin	9 (69.2)	0	0	0
Fondaparinux	1 (7.7)	0	0	0
Statin	1 (7.7)	3 (37.5)	0	0

Of the 33 subjects recruited, 6 discontinued the study prior to the 6-week study visit. Reasons for discontinuation included: gastrointestinal upset after starting HCQ (2), subject decided not to start HCQ (1), SLE flare requiring initiation of immunosuppressive medication (2), and lost to follow up (1). An additional 2 subjects discontinued the study after the 6-week study visit but before the 12-week visit due to new pregnancy (1) and lost to follow up (1). There were no thrombotic events during the study period.

Baseline data

We analyzed baseline data for all 33 subjects. Baseline values for the AnxA5-RA, aDI- β_2GPI, and APC resistance differed significantly between aPL-positive and aPL-negative subjects, while baseline D-dimer values did not (Table 2).

Table 2. Baseline Values of Non-Criteria aPL Tests for aPL-positive and aPL-negative Subjects (n=33)

	aPL (+), n: 21	aPL (-) SLE, n: 12	p	Reference Range
AnxA5-RA (%) (Mean ± SD)	134.1 ± 15.6	168.3 ± 21.9	<0.0001	Negative ≥153 Borderline 140-152.9 Positive <140
aDI-β_2GPI (mg/L) (Mean ± SD)	4.40 ± 4.38	0.76 ± 0.44	0.0002	Negative ≤1.66 Borderline 1.66-2.29 Positive >2.29
APC resistance ratio	2.7 ± 0.6*	3.2 ± 0.6	0.0085	Abnormal <2.0
D-dimer (µg/mL) (Mean ± SD)	1.77 ± 0.67	1.60 ± 1.35	0.1444	Normal 0.8-2.3

AnxA5-RA: Annexin-A5 resistance assay; aDI-β_2GPI: anti-domain-I β_2-glycoprotein-I antibody; APC: activated protein C; SD: standard deviation

*n=16 for aPL-positive patients

Follow-up data

We analyzed follow-up data for the 17 aPL-positive subjects who completed 12 weeks of study follow up. As shown in Table 3, values for AnxA5-RA, aDI-β_2GPI, APC resistance, and D-dimer did not change significantly from baseline to 12 weeks in aPL-positive subjects taking HCQ. There was also no significant change in LAC status, aCL or aβ_2GPI titers (data not shown).

Table 3: Comparison of Baseline and Week 12 Values for Non-criteria aPL Tests in aPL-positive Subjects (n=17)

	Baseline	12 weeks	p	Reference Range
AnxA5-RA (%) (Mean ± SD)	136.6.1 ± 16.0	143.96 ± 25.6	0.2435	Negative ≥153 Borderline 140-152.9 Positive <140
aDI-β_2GPI (mg/L) (Mean ± SD)	4.45 ± 4.80	4.01 ± 4.40	0.9265	Negative ≤1.66 Borderline 1.66-2.29 Positive >2.29
APC resistance ratio*	2.69 ± 0.63	2.52 ± 0.92	0.7002	Abnormal <2.0
D-dimer (µg/mL) (Mean ± SD)	1.73 ± 0.72	1.58 ± 0.72	0.1743	Normal 0.8-2.3

AnxA5-RA: Annexin-A5 resistance assay; aDI-β_2GPI: anti-domain-I β_2-glycoprotein-I antibody; APC: activated protein C; SD: standard deviation

* n = 14

We further analyzed the subpopulation of APS subjects to assess for a change in these test results from baseline to week 12. Twelve of the 13 subjects with APS completed 12 weeks of study follow up. As shown in Table 4, values for AnxA5-RA, aDI-β_2GPI, APC resistance, and D-dimer did not change from baseline to 12 weeks in subjects with APS taking HCQ.

Table 4: Comparison of Baseline and Week 12 Values for Non-criteria aPL Tests in Subjects with APS (n=12)

	Baseline	12 weeks	p	Reference Range
AnxA5-RA (%) (Mean ± SD)	133.8 ± 17.0	145.0 ± 29.0	0.151	Negative ≥153 Borderline 140-152.9 Positive <140
aDI-β_2GPI (mg/L) (Mean ± SD)	5.0 ± 4.8	4.2 ± 4.1	0.791	Negative ≤1.66 Borderline 1.66-2.29 Positive >2.29
APC resistance ratio*	2.4 ± 0.6	2.5 ± 0.6	0.156	Abnormal <2.0
D-dimer (µg/mL) (Mean ± SD)	1.80 ± 0.83	1.74 ± 0.77	0.5186	Normal 0.8-2.3
AnxA5-RA: Annexin-A5 resistance assay; aDI-β_2GPI: anti-domain-I β_2-glycoprotein-I antibody; APC: activated protein C; SD: standard deviation * n = 9				

Discussion

aPL-mediated interference with the AnxA5 anticoagulant layer over cells plays a role in the pathogenesis of APS,[12-14] and patients with aPL have reduced AnxA5 anticoagulant activity. HCQ reverses the effects of aPL and restores AnxA5 expression on cell surfaces.[15,16]

In this study, subjects with aPL have reduced AnxA5 anticoagulant activity and elevated titers of aDI-β_2GPI. Others have remarked on the particular importance of aDI-β_2GPI in the pathogenesis of APS as domain I is a major antigenic target for thrombogenic aPL.[17] Our findings support the use of the AnxA5-RA and aDI-β_2GPI tests to detect potentially pathogenic aPL.

Our study confirms the observations of previous studies by showing that aPL-positive subjects have abnormal baseline AnxA5-RA measurements compared to aPL-negative control subjects (SLE-only individuals in this study). We did not demonstrate a significant difference in the baseline AnxA5-RA between asymptomatic aPL-positive individuals and those with thrombotic APS, suggesting that this assay does not distinguish those with historical thrombosis from those without; follow-up of those asymptomatic aPL-positive individuals with abnormal AnxA5-RA are warranted to determine if the assay is of those individuals who will develop future thrombosis.

We did not demonstrate a change in the AnxA5-RA over a 12 week time period in subjects taking HCQ. There are several possible explanations. One is that 12 weeks is not a sufficient period of time to detect a change in the assay. Lack of medication adherence is another possibility. Although we considered that the dosing of HCQ may not have resulted in therapeutic levels of the drug, we did not measure HCQ levels in

this study. Finally, this pilot study was not powered to detect a change within groups over time, but was intended to be hypothesis-generating for future studies. Though not definitive, our findings suggest that HCQ may not act through an AnxA5 resistance mechanism.

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