CONCISE REPORT

A prospective open-label pilot study of fluvastatin on proinflammatory and prothrombotic biomarkers in antiphospholipid antibody positive patients

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ABSTRACT

Objective To determine if proinflammatory and prothrombotic biomarkers are differentially upregulated in persistently antiphospholipid antibody (aPL)-positive patients, and to examine the effects of fluvastatin on these biomarkers.

Methods Four groups of patients (age 18–65) were recruited: (a) primary antiphospholipid syndrome; (b) systemic lupus erythematosus (SLE) with antiphospholipid syndrome (APS) (SLE/APS); (c) persistent aPL positivity without SLE or APS (Primary aPL); and (d) persistent aPL positivity with SLE but no APS (SLE/aPL). The frequency-matched control group, used for baseline data comparison, was identified from a databank of healthy persons. Patients received fluvastatin 40 mg daily for 3 months. At 3 months, patients stopped the study medication and they were followed for another 3 months. Blood samples for 12 proinflammatory and prothrombotic biomarkers were collected monthly for 6 months.

Results Based on the comparison of the baseline samples of 41 aPL-positive patients with 30 healthy controls, 9/12 (75%) biomarkers (interleukin (IL)-6, IL1β, vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF)-α, interferon (IFN)-α, inducible protein-10 (IP10), soluble CD40 ligand (sCD40L), soluble tissue factor (sTF) and intracellular cellular adhesion molecule (ICAM)-1) were significantly elevated. Twenty-four patients completed the study; fluvastatin significantly and reversibly reduced the levels of 6/12 (50%) biomarkers (IL1β, VEGF, TNFα, IP10, sCD40L and sTF).

Conclusions Our prospective mechanistic study demonstrates that proinflammatory and prothrombotic biomarkers, which are differentially upregulated in persistently aPL-positive patients, can be reversibly reduced by fluvastatin. Thus, statin-induced modulation of the aPL effects on target cells can be a valuable future approach in the management of aPL-positive patients.

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disorder of thromboses and pregnancy losses associated with persistent antiphospholipid antibodies (aPL) (lupus anticoagulant (LA) test, anticardiolipin antibodies (aCL) and anti-β_2 glycoprotein-I antibodies (aβ_2GPI)).[1] aPL can occur in otherwise healthy individuals as well as in 30%–40% of systemic lupus erythematosus (SLE) patients. aPL-mediated clinical events occur due to complex interaction of proinflammatory and prothrombotic cells. *First*, aPL increase endothelial cell (EC) expression of the cellular adhesion molecules (CAMs) such as intracellular CAM-1 (ICAM-1), vascular CAM-1 (VCAM-1) and E-selectin (E-sel).[2–6] *Second*, tissue factor (TF) upregulation is an important mechanism of the prothrombotic effects of aPL.[7–9] *Third*, aPL induce significant increase in proinflammatory cytokines (interleukin (IL)-6, IL-8 and tumour necrosis factor-α (TNF-α)) on EC.[8 9] Fluvastatin diminishes aPL-mediated upregulation of adhesion molecules and TF in vitro in ECs, as well as the in vivo thrombogenic and proinflammatory effects of aPL in mice.[10–12]

Given the relationship between thrombosis and increased expression of CAMs, TF activity and proinflammatory cytokines in APS, we hypothesise that patients with persistently positive aPL have increased levels of proinflammatory and prothrombotic biomarkers when compared with healthy controls, and fluvastatin treatment for 3 months decreases significantly and reversibly the level of these biomarkers.

METHODS

Study design

The primary objective of this open-label prospective pilot intervention trial was to determine if proinflammatory and prothrombotic biomarkers are differentially upregulated in persistently aPL-positive patients with or without SLE. The secondary objective was to determine the effects of fluvastatin on prothrombotic and proinflammatory biomarkers in aPL-positive patients with or without SLE.

Study population and inclusion/exclusion criteria

Four groups of patients (age 18–65) were recruited: (a) primary APS (PAPS); (b) SLE with APS (SLE/APS); (c) persistent aPL positivity without SLE or APS (primary aPL); and (d) persistent aPL positivity with SLE but no APS (SLE/aPL). SLE was defined based on the American College of Rheumatology classification criteria.[13] APS was defined based on the updated Sapporo classification

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criteria. Positive aPL was defined as persistently (at least 12 weeks apart) positive LA test, aCL≥40 GPL/MPL and/or aβ$_2$GPI≥20 SGU/SMU.[1]

Exclusion criteria were age less than 18 years old; pregnancy; statin or any other cholesterol-lowering agent within three months prior to the screening; underlying liver or muscle disease; chronic renal failure requiring dialysis; active infections requiring antibiotics; systemic autoimmune disease other than SLE; routine use of non-steroidal anti-inflammatory drug (NSAID); use of prednisone >10 mg/day; use of immunosuppressive/s (except hydroxychloroquine) within 1 month prior to the screening; use of biologic agents within 6 months prior to the screening; treatment with protease inhibitors, rifampin, rifabutin, cholestyramine, fluconazole, itraconazole, ketoconazole, synercid, delavirdine, erythromycin or clarithromycin within 1 week prior to screening; history of an allergic reaction to statins; and active illegal drug use or alcohol abuse within the last 52 weeks.

The frequency-matched control groups (n: 30) were identified from a databank of healthy persons (no autoimmune or inflammatory diseases) at The University of Texas Medical Branch (UTMB).

Study interventions

All subjects had provided informed consent approved by the Internal Review Boards at UTMB and Hospital for Special Surgery (ClinicalTrials.gov NCT00674297). Baseline data collection included demographics, general and aPL-specific medical history, medications, blood for specialised outcome measures and safety outcome measures (aspartate transaminase (AST), alanine aminotransferase (ALT), creatinine phosphokinase (CPK) and urine pregnancy test for pre-menopausal patients). Within a week of the screening visit, all patients were started on fluvastatin 40 mg daily for 3 months. At 3 months, patients were instructed to stop the study medication and they were followed for another 3 months. Blood samples for specialised outcome measures were collected at the baseline visit, and one, two, three, four, five and six months. Blood samples for safety outcome measures were collected at the baseline visit and 2 months after. A window period of ±4 days was allowed for each study visit.

After the enrolment, if an immunosuppressive medication and/or >10 mg of prednisone were indicated for the treatment of any disease activity, patients were withdrawn from the study. Patients were instructed not to use NSAIDs regularly during the study period, and any occasional NSAID use was recorded during the monthly study visits. If patients reported the use of an NSAID for more than seven consecutive days, they were withdrawn from the study.

Specialised outcome measures

An in-house ELISA and a commercial ELISA (INOVA Diagnostics, San Diego, California, USA) were used to measure aCL and aβ$_2$GPI, respectively. LA activity was determined as recommended by the International Society on Thrombosis and Haemostasis Subcommittee.[14]

The MILLIPLEX MAP Human Cytokine/Chemokine Panel Assay (Millipore, Billerica, Massachusetts, USA), which uses Luminex xMAP technology, was used to determine levels of IL-1β, IL-6, IL-8, TNF-α, interferon-α (IFN)-α, IP10, vascular endothelial growth factor (VEGF) and sCD40L. Intercellular adhesion molecule 1, E-sel and VCAM-1 serum levels were assessed using a commercial ELISA kit (R&D systems, Minneapolis, Minnesota, USA). Plasma samples were used to detect soluble TF (sTF) using a chromogenic assay (American Diagnostics, Stamford, Connecticut, USA). Cut-offs for the cytokines, cellular adhesion molecules and TF were determined as the 95th percentile of 30 healthy controls for each assay.

Safety outcome measures

The safety of fluvastatin was evaluated immediately after starting the medication at 1, 2, 3, 4, 5 and 6 months. An adverse event (AE) was defined as any untoward medical occurrence in a study patient regardless of causality assessment. A serious AE was defined as one occurring at any dose that met one or more of the following criteria: death; life-threatening; requiring or prolonging inpatient hospitalisation; disabling; or resulting in a congenital anomaly/birth defect.

In case of persistent AST, ALT and/or CPK elevations, that is, more than two times the normal values twice in one week, the patient was withdrawn from the study.

Statistical analyses

The Kruskal–Wallis test was used to compare the levels of biomarkers in aPL-positive subjects to the controls. Spearman test was used to analyse the significance of monthly changes in biomarker levels and to correlate the levels of the biomarkers in the different subgroups of patients before and after treatment.

RESULTS

Patients demographics

Forty-one aPL positive patients (female: 31 (74%), mean age: 43.5±12.5, Caucasian: 28 (68%); primary APS: 18, SLE/APS: 7, primary aPL: 9 and SLE/aPL: 7) were enrolled and their baseline samples were compared with 30 frequency-matched healthy controls (female: 25 (85%); mean age: 43.5±12.5). All APS patients had history of thrombosis except two SLE/APS patients who had only obstetric APS. In patients with vascular events, the mean time between the event and the first blood collection was 54.6±79.2 months (range 1–240 months). Out of the 22 patients that were not on anticoagulation at the time of the enrolment, 16 (73%) were LA test positive. Twenty-three (56%) patients were on hydroxychloroquine, 7 (17%) on prednisone (mean dose: 5.8±1.1) and 19 (56%) on low-dose aspirin.

Baseline biomarkers of patients compared to healthy controls

Table 1 demonstrates the median levels and IQR of specific outcome measures. While the serum or plasma levels of all the biomarkers were above the cut-off of each assay, in 20%–100% of the aPL-positive subjects, IL-6, IL-1β, VEGF, TNF-α, IFN-α, IP-10, sCD40L, sTF and sICAM-1 were significantly elevated compared to healthy controls. Many of the biomarkers correlated well among each other, the most significant being TNFα and IL8 ($r=0.848$, $p<0.001$) and IL6 and VEGF ($r=0.506$, $p=0.001$).

Based on a subgroup analysis, the levels of: (a) IL-8, TNF-α and IP10 were significantly higher in PAPS, SLE/APS and SLE/aPL when compared with primary aPL; (b) VEGF, sICAM-1 and sVCAM-1 were significantly higher in PAPS when compared with the other groups; and (c) sTF and sCD40L were elevated in all subgroups when compared with controls (table 1).

Effect of fluvastatin on specialised outcome measures in persistently aPL-positive patients

Of 41 patients recruited, 24 completed the study (mean age: 44.6±13.6; female: 70%; primary APS: 8, SLE/APS: 7, primary aPL: 5; SLE/aPL: 4). Nine (43%) patients were on

Table 1 Levels of proinflammatory and prothrombotic biomarkers in antiphospholipid antibody (aPL) positive patients compared with healthy controls: combined and subgroup analysis

Biomarkers Median (IQR)	Controls n: 30	Combined n: 41	PAPS n: 18	SLE/APS n: 7	SLE/aPL n: 7	Primary aPL n: 9
IL6 (pg/mL)	0.7 (0.0)	38.0* (47.3)	31.2* (48.4)	12.2* (103.5)	2.7* (76.8)	0.4 (0.7)
IL1β (pg/mL)	0.3 (0.1)	4.7* (25.1)	3.0* (7.7)	11.4* (20.1)	0.5 (5.1)	0.3 (0.6)
IL8 (pg/mL)	27.4 (30.0)	42.6 (43.9)	24.5** (48.4)	27.4** (53.7)	21.6** (48.9)	7.2 (50.4)
VEGF (pg/mL)	88.3 (85.2)	225.1* (318.4)	242.2*** (399.6)	109.1 (348.4)	67.2 (499.4)	74.6 (158.3)
TNFα (pg/mL)	0.5 (0.0)	29.9* (27.3)	21.5*,** (50.5)	11.6*,** (24.1)	53.9** (62.5)	8.9* (15.7)
IFNα (pg/mL)	0.1 (10.2)	12.9* (115.7)	10.1 (88.4)	0.3 (367.1)	13.2 (558.5)	0.3 (512.2)
IP10 (pg/mL)	96.2 (58.0)	584.4* (551.8)	427.2*,** (569.8)	656.2*,** (454.8)	472.5*,** (690.5)	249.7 (698.9)
sCD40L (pg/mL)	16.4 (14.6)	230.1* (2730.8)	276.5* (676.0)	145.6* (5159.8)	76.9* (970.0)	149.7* (17 035.6)
sTF (pM)	13.0	134.0* (206.0)	153.6* (381.0)	329.2* (447.0)	102.1* (177.0)	190.4* (139.0)
sICAM-1 (ng/mL)	9.5	151.3* (300.2)	281.6*,*** (406.8)	55.1* (70.3)	2.8 (32.9)	163.5 (341.7)
sVCAM-1 (ng/mL)	33.7	41.9 (444.2)	1128.4*,*** (1152.8)	156.4 (19.3)	41.1 (27.2)	321.3 (1015.1)
sE-sel (ng/mL)	10.1	14.1 (27.5)	27.7* (36.7)	14.7 (20.4)	4.1 (21.5)	10.9 (26.4)
# of biomarker elevated*		9/12	9/12	7/12	4/12	3/12

The mean levels of aCL IgG (53.3 GPL), aCL IgM (30.9 MPL), aCL IgA (13.7 APL), antiB2GPI IgG (42.3 SGU), antiB2GPI IgM (44.7 SMU) and antiB2GPI IgA (29.8 SAU) were significantly higher compared to healthy controls ($p<0.05$).
*$p<0.05$ compared with controls, **$p<0.05$ compared with primary aPL patients, ***$p<0.05$ in PAPS versus all other patient groups.
APS, antiphospholipid syndrome; IFNα, interferon α; IL, interleukin; IP10, inducible protein 10; PAPS, primary antiphospholipid syndrome; sCD40L, soluble CD 40 ligand; sEsel, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule; SLE, systemic lupus erythematosus; sTF, soluble tissue factor; sVCAM-1, soluble vascular cell adhesion molecule 1; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor.

anticoagulation, 15 (61%) on hydroxychloroquine, four on prednisone (mean dose: 4.5±1.1) and 10 (41%) on low-dose aspirin. The early withdrawal reasons for 15 patients were: five lost to follow-up or refused treatment after the baseline visit; four stopped treatment due to myalgia; three wanted to continue fluvastatin after 3 months; one did not receive the treatment due to baseline elevated liver function tests; and one stopped treatment due to insomnia. AEs that occurred in 8 out of 38 (21%) patients during a mean of 74±26 days of fluvastatin treatment were: arthralgia (n: 1), lupus flare (n: 1), myalgia with high CPK (n: 1), myalgia with normal CPK (n: 3), recurrent deep vein thrombosis (DVT) (n: 1), headache (n: 1) and insomnia (n: 1). There were no serious AEs.

Figure 1 shows the effects of fluvastatin on the biomarkers within 3 months of fluvastatin treatment. The levels of 8/12 (66%) biomarkers (IL-6, IL-1β, VEGF, TNF-α, IFN-α, IP-10, sCD40L and sTF) significantly decreased with fluvastatin; mean maximum reduction of biomarkers was achieved between 30

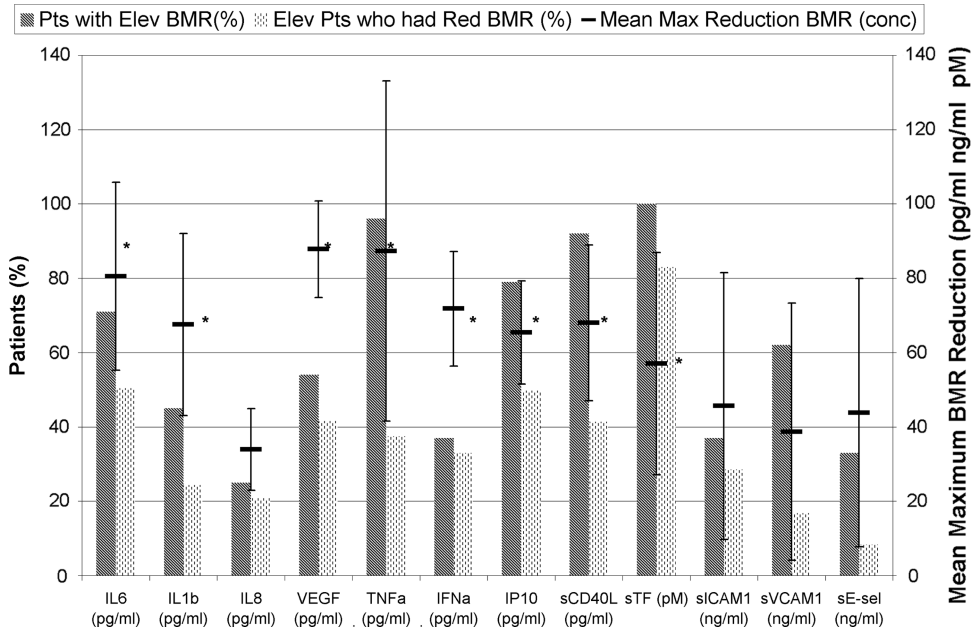

Figure 1 Effects of fluvastatin on proinflammatory and prothrombotic biomarkers (BMR) in antiphospholipid antibody-positive patients (pts). The percentage of patients with elevated (elev) BMR levels at baseline (■) and with subsequent reduced (red) levels following fluvastatin (■) is shown on the left primary vertical axis. The mean (±SD) maximum (max) level of BMR concentration (conc) reduction following fluvastatin is shown on the right secondary vertical axis (bars (Ξ). *$p<0.05$. IL, interleukin; IFN, interferon; IP10, inducible protein 10; sCD40L, soluble CD 40 ligand; sEsel, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule; sTF, soluble tissue factor; sVCAM-1, soluble vascular cell adhesion molecule 1; TNFα, tumour necrosis factor α; and VEGF, vascular endothelial growth factor.

Table 2 Proinflammatory and prothrombotic biomarkers in aPL-positive patients after stopping fluvastatin

Biomarker (BMR)	# of Patients with increased BMR after stopping fluvastatin (%)	Mean±SD maximum BMR increase after stopping fluvastatin	Mean time (days) to maximum BMR increase after stopping fluvastatin
IL6 (pg/mL)	9/12 (75%)	56.7±34.5	35±12
IL1β (pg/mL)	5/6 (83%)	89.0±23.5*	43±15
IL8 (pg/mL)	3/5 (60%)	45.6±34.1	60±10
VEGF (pg/mL)	5/10 (50%)	57.8±28.5*	48±15
TNFα (pg/mL)	6/9 (67%)	90.3±4.5*	58±17
IFNα (pg/mL)	6/8 (75%)	56.7±21.0	60±20
IP10 (pg/mL)	8/12 (67%)	87.5±14.5*	55±15
sCD40L (pg/mL)	9/10 (90%)	90.6±4.3*	45±15
sTF (pM)	13/20 (65)	80.4±10.3*	50±12
sICAM-1 (ng/mL)	1/7 (14)	23.4±12.0	60
sVCAM-1 (ng/mL)	3/4 (75)	47.6±10.4	46±15
sE-sel (ng/mL)	0/2 (0)	N/A	N/A

*p<0.05.
IFNα, interferon α; IL, interleukin; IP10, inducible protein 10; N/A, not applicable; sCD40L, soluble CD 40 ligand; sEsel, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule; sTF, soluble tissue factor; sVCAM-1, soluble vascular cell adhesion molecule 1; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor.

and 70 days of fluvastatin treatment. More than 80% of the subjects with elevated levels of sTF, TNF-α and IFN-α showed a significant reduction with fluvastatin.

Table 2 shows the effects of stopping fluvastatin on the biomarkers during the second half of the study. The levels of 6/8 (75%) biomarkers (IL-1β, VEGF, TNF-α, IP-10, sCD40L and sTF) significantly increased after stopping the fluvastatin treatment; 14%–90% of the patients with fluvastatin-induced reduction of the biomarkers showed an increase in the levels of the biomarker.

Clinical observations

A 36-year-old woman with SLE/APS developed diffuse arthritis at week 8. The baseline IL-6, IL-1β, IL-8, TNF-α, IP-10, sCD40L and sVCAM-1 levels were significantly elevated when compared with controls; a significant reduction of IFN-α (75%), IL-6 (82%), IL-8 (84%), TNF-α (65%) and VEGF (53%) occurred after 4 weeks of fluvastatin. At week 8, when the patient had a lupus flare, there was a significant increase in these biomarkers (IFN-α (500%), IL-6 (226%), IL-8 (246%), TNF-α (837%) and VEGF (67%)) compared to week 4; in addition, IL-1β and sTF were significantly increased compared to baseline (186% and 75%, respectively) even if the change between baseline and week 4 was not significant.

A 30-year-old man with SLE/APS developed recurrent DVT at week 12. The baseline IFNα, TNFα, IP10 and IL6 levels were elevated when compared with controls; a significant reduction of IL6, IFNα, sTF and IP10 was observed after 4 weeks of fluvastatin. At week 12, when the patient developed a recurrent DVT, the IL6, TNFα, IP10 and sTF levels were significantly elevated.

DISCUSSION

Our prospective mechanistic study investigating the effect of fluvastatin on proinflammatory and prothrombotic biomarkers demonstrated that IL-6, IL-1β, VEGF, TNF-α, IFN-α, IP-10, sCD40L, sTF and sICAM are differentially upregulated in aPL-positive patients with or without vascular events and/or SLE; the majority of these biomarkers (IL-1β, VEGF, TNF-α, IP-10, sCD40L and sTF) can be significantly and reversibly reduced by fluvastatin.

A commonly accepted theory for thrombosis in aPL-positive patients is that aPL increase the thrombophilic threshold as the 'first hit' (induce a proinflammatory/thrombotic phenotype via the cytokines and chemokines), and then clotting takes place only when a 'second hit' (infection, inflammation, surgical procedures or use of estrogens) exists.[15–21] Our findings, especially elevated levels of sTF and sCD40L in persistently aPL-positive patients independent of the APS or SLE diagnosis, strengthen this theory and suggest that these biomarkers may have a predictive role in aPL-positive patients for the development of APS or SLE.

Fluvastatin prevents the expression of cellular adhesion molecules, TF and IL-6 in aPL-treated ECs in vitro.[11] In the only human mechanistic study published, using a proteomic analysis, López-Pedrera *et al* showed that inflammatory proteins can be reversed in aPL-positive patients following 1 month of daily 20 mg fluvastatin.[21] In our study, we extended the treatment with fluvastatin to 3 months and also monitored biomarkers for an additional 3 months after discontinuation of the treatment. All the biomarkers were reduced by fluvastatin within 2 months, suggesting that the potential thrombosis risk in persistently aPL-positive patients also decreases within the same time frame. Furthermore, the prospective and self-controlled nature of the study allowed us to demonstrate the rebound elevation of the majority of the biomarkers after cessation of the therapy.

Interestingly, one patient experienced a lupus flare with concomitant and significant elevation of selected proinflammatory and prothrombotic markers indicating that these biomarkers are sensitive to fluctuations in disease activity despite statin treatment. This observation is important in the sense that the beneficial effects of statins in aPL-positive patients can be mitigated in the setting of a lupus flare.

Our study has several limitations. First, aPL-positive patients with diverse clinical manifestations were included in the study; the cytokine pattern of our patients could therefore reflect, at least in part, differences in the molecular mechanisms of clinical phenotypes. Second, the sample size is relatively small, and thus, we were not able to perform a subgroup analysis of the effects of fluvastatin on the biomarkers. Third, different statins may have diverse pleiotropic effects; given that all the in vitro/vivo studies in APS were completed using fluvastatin, we used fluvastatin in this study for consistency purposes. And lastly, our study cannot fully elucidate the association between other comorbidities and change in biomarker levels.

In summary, our prospective mechanistic pilot study with frequency-matched controls demonstrates that proinflammatory and prothrombotic biomarkers, which are differentially upregulated in aPL-positive patients with or without vascular events and/or SLE, can be reversibly reduced by fluvastatin. Thus, statin-induced modulation of the aPL effects on target cells can be a valuable future approach in the management of aPL-positive patients.

Contributors DE, SSP and JV contributed to the conception and design of the study. DE, SSP and RW contributed to the writing of the manuscript. JV, RW, VLM, EBG, EP, PRL, ALC, LAM-M and EBG contributed to analysis and interpretation of data and critical review of the manuscript. All authors contributed to the final approval of the manuscript.

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Competing interests None.

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