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Potent reduction of plasma lipoprotein (a) with an antisense oligonucleotide in human subjects does not affect ex vivo fibrinolysis[S]

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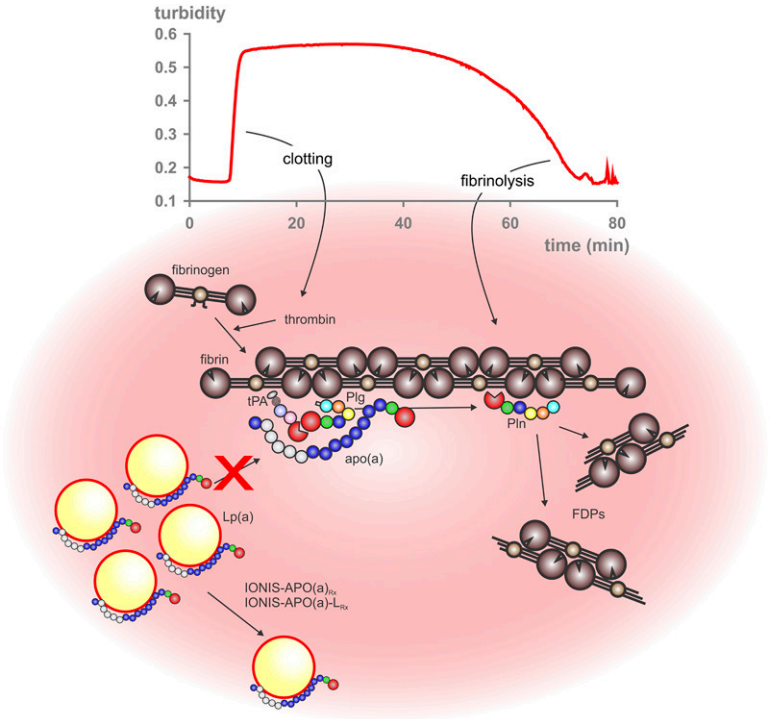
Abstract **It is postulated that lipoprotein (a) [Lp(a)] inhibits fibrinolysis, but this hypothesis has not been tested in humans due to the lack of specific Lp(a) lowering agents. Patients with elevated Lp(a) were randomized to antisense oligonucleotide [IONIS-APO(a)$_{Rx}$] directed to apo(a) ($n = 7$) or placebo ($n = 10$). Ex vivo plasma lysis times and antigen concentrations of plasminogen, factor XI, plasminogen activator inhibitor 1, thrombin activatable fibrinolysis inhibitor, and fibrinogen at baseline, day 85/92/99 (peak drug effect), and day 190 (3 months off drug) were measured. The mean ± SD baseline Lp(a) levels were 477.3 ± 55.9 nmol/l in IONIS-APO(a)$_{Rx}$ and 362.1 ± 89.9 nmol/l in placebo. The mean± SD percentage change in Lp(a) for IONIS-APO(a)$_{Rx}$ was −69.3 ± 12.2% versus −5.4 ± 6.9% placebo ($P < 0.0010$) at day 85/92/99 and −15.6 ± 8.9% versus 3.2 ± 12.2% ($P = 0.003$) at day 190. Clot lysis times and coagulation/fibrinolysis-related biomarkers showed no significant differences between IONIS-APO(a)$_{Rx}$ and placebo at all time points. Clot lysis times were not affected by exogenously added Lp(a) at concentrations up to 200 nmol/l to plasma with very low (12.5 nmol/l) Lp(a) levels, whereas recombinant apo(a) had a potent antifibrinolytic effect. In conclusion, potent reductions of Lp(a) in patients with highly elevated Lp(a) levels do not affect ex vivo measures of fibrinolysis; the relevance of any putative antifibrinolytic effects of Lp(a) in vivo needs further study.**



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Abbreviations: HLD-C, HDL cholesterol; LDL-C, LDL cholesterol; Lp(a), lipoprotein (a); PAI-1, plasminogen activator inhibitor 1; TAFI, thrombin activatable fibrinolysis inhibitor; tPA, tissue-type plasminogen activator.

This trial was registered at clinicaltrials.gov as NCT02160899 and NCT02414594.

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Lipoprotein(a) [Lp(a)] is a genetic risk factor for CVD and calcific aortic valve stenosis. Lp(a) is thought to mediate clinical events by three main mechanisms: proatherogenic effects via its LDL cholesterol (LDL-C) moiety (1, 2), proinflammatory effects via its content of oxidized phospholipids (3–5), and antifibrinolytic effects via its apolipoprotein(a) [apo(a)] component (6).

Lp(a) has high homology (75–99%) to plasminogen but lacks protease activity and therefore has been hypothesized to inhibit fibrinolysis and mediate prothrombotic potential. This hypothesis has been supported by in vitro/ex vivo studies primarily using free apo(a), of which little if any is present in plasma in vivo, rather than purified Lp(a) (7–12). More recently, several association studies using genetic instruments related to *LPA*, the gene encoding apo(a), have suggested elevated Lp(a) levels are not associated with deep venous thrombosis (13, 14). These studies argue against a direct role of Lp(a) in thrombosis, except perhaps when there is a second concomitant prothrombotic etiology such as Factor V Leiden in pediatric stroke (15) or elevated homocysteine levels (16). Furthermore, elevated Lp(a) levels are causally associated with calcific aortic valve stenosis, where thrombosis is not a part of the clinical phenotype (17). On the other hand, whether Lp(a) might directly contribute to the thrombotic sequelae of arterial plaque rupture has been difficult to assess because of the inability to disentangle such effects from underlying atherosclerotic disease.

The role of Lp(a) lowering on its effects on fibrinolysis in humans has not been previously investigated due to the lack of specific Lp(a) lowering agents. IONIS-APO(a)$_{Rx}$ and IONIS-APO(a)-L$_{Rx}$, having the same sequence as IONIS-APO(a)$_{Rx}$ but additionally containing the hepatocyte targeting moiety N-acetylgalactosamine, are second-generation antisense oligonucleotides directed to apo(a). In phase I and II clinical trials they have been demonstrated to lower mean Lp(a) levels by 70% to 92% (18, 19). This degree of reduction in Lp(a) is much higher than has been achieved by agents that are not specific for Lp(a) lowering such as niacin (20) and antibody inhibitors of proprotein convertase subtilisin/kexin type 9 (21).

In this study, we evaluated whether substantial lowering of elevated Lp(a) results in an improvement in fibrinolytic potential using ex vivo clot lysis assays and measuring coagulation/fibrinolysis biomarkers.

MATERIALS AND METHODS

Study subjects

Study subjects were enrolled from three trials assessing antisense oligonucleotides directed to apo(a). The first study was a phase 1 study that used ISIS-APO(a)$_{Rx}$ in healthy volunteers (18), the second study was a phase 2 study in subjects with elevated Lp(a) with the same drug [but relabeled as IONIS-APO(a)$_{Rx}$] (19), and the third study was a phase 1 study in healthy volunteers with elevated Lp(a) that used IONIS-APO(a)L$_{Rx}$, an N-acetylgalactosamine-conjugated antisense oligonucleotide to apo(a) (19).

Ex vivo clot lysis time was measured in baseline samples from healthy subjects (n = 16) enrolled in a phase 1 study of ISIS-APO(a)$_{Rx(18)}$ to ascertain the normal range of clot lysis times. Subjects were healthy adults aged 18–65 years with a BMI <32 kg/m^2 and Lp(a) levels >25 nmol/l. In the phase 2 study, patients (n = 64) with Lp(a) >125 nmol/l (>50 mg/dl) were randomized to placebo or IONIS-APO(a)$_{Rx}$, dosed subcutaneously at 100 mg weekly for 1 month, 200 mg weekly for the second month, and 300 mg weekly for the third month (ClinicalTrials.gov identifier: NCT02160899) (18). In a subset of these patients with the highest Lp(a) levels [10 placebo, 7 IONIS-APO(a)$_{Rx}$] ex vivo plasma clot lysis time and antigen concentrations of plasminogen, factor XI, plasminogen activator inhibitor 1 (PAI-1), thrombin activatable fibrinolysis inhibitor (TAFI), fibrinogen and oxidized phospholipid content on apoB-100, apo(a), and plasminogen (22) were measured at baseline, day 85/92/99 (representing peak drug effect), and day 190 (3 months off drug). Blood sampling at day 85/92/99 was used, as not all patients had blood sampling on all 3 days. To assess whether the findings were drug-specific, ex vivo clot lysis assays in three otherwise healthy human volunteers with highly elevated Lp(a) levels enrolled in the IONIS-APO(a)L$_{Rx}$ trial (ClinicalTrials.gov identifier: NCT02414594) (19). Approval for the study was obtained at local institutional review boards, and subjects gave written informed consent to participate.

Ex vivo clot formation and clot lysis assays

Ex vivo clot formation and clot lysis assays were performed in 33% diluted patient plasma collected in EDTA added to wells containing final concentrations of 10 mmol/l $CaCl_2$, 0.01% thromboplastin-DS, 10 μmol/l phosphatidylserine-phosphatidylcholine (80:20) vesicles, and 1 nmol/l tissue-type plasminogen activator (tPA). Clot formation and clot lysis times were monitored turbidometrically at 37°C in a plate-reading spectrophotometer (**Fig. 1**). In some experiments, plasma from a single donor with low Lp(a) was used, and clots were supplemented with increasing doses of Lp(a), purified as described (23), or the following recombinant variants of apo(a) were used: 17-kringle (17K), 17KΔLBS10, 30K, 14K, 12K, and 6K (24).

Laboratory analyses

Lp(a) concentrations were measured in nmol/l, and apo(a) isoform sizes were measured at the Northwest Lipid Metabolism and Diabetes Research Laboratories at the University of Washington (Seattle, WA) as previously described (19). Plasma concentrations of apoB-100, total cholesterol, LDL-C, HDL cholesterol (HLD-C), VLDL cholesterol, and triglycerides were measured with commercially available assays (Medpace Reference Laboratories) (19). Lp(a) cholesterol was estimated by converting the Lp(a) particle number in nmol/l to Lp(a) in mg/dl and dividing by 2.4; this value was then multiplied by 0.3 (19). Antigen concentrations of Factor XI (Affinity Biologicals), fibrinogen (Enzo Life Sciences), PAI-1 (Life Technologies), and TAFI (Affinity Biologicals) were measured with commercial assays. Plasminogen, OxPL-apoB, OxPL-apo(a), and OxPL-plasminogen were measured with in-house assays at University of California San Diego as previously described (22).

Statistical analysis

Continuous data are expressed as means ± SDs; the comparisons between the two treatment arms [IONIS-APO(a)$_{Rx}$ treatment and placebo] in the IONIS-APO(a)$_{Rx}$ trial were performed using the two-sample t-test. If data departed substantially from normality, the Wilcoxon rank-sum test was used. Categorical data are expressed as frequencies (number of patients in category) and percentages; the comparisons between the two treatment arms in the IONIS-APO(a)$_{Rx}$ trial were performed using Fisher's exact

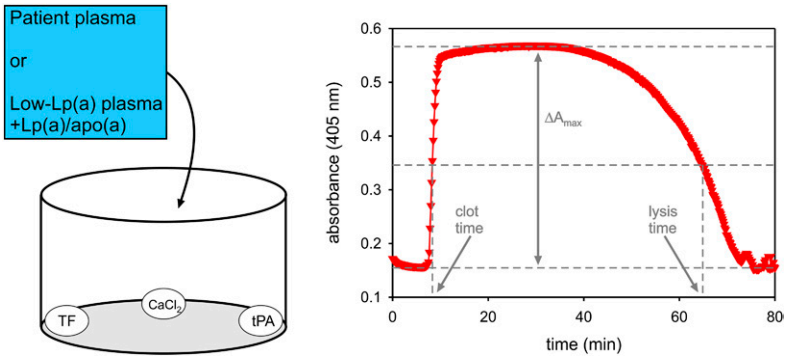

Fig. 1. Methodology of clot lysis assay. Left: Design of the ex vivo clot lysis assay, where plasma in the presence or absence of purified Lp(a) or apo(a) is added to the wells of a 96-well plate containing microaliquots of tissue factor (TF) and $CaCl_2$ to initiate clot formation and tPA to initiate fibrinolysis. Right: Representative lysis profile showing how clot time and lysis time are derived from the midpoints of the increase and decrease in clot turbidity, respectively. In all cases the clot time is subtracted from the lysis time.

test. Correlations between lysis time and baseline plasma Lp(a) were assessed by Spearman's rank correlation coefficient, and differences in lysis time between Lp(a) and recombinant apo(a) variants were assessed by ANOVA with Lp(a)/apo(a) or apo(a) variants and their concentrations as the factors and adjusted using Tukey's post hoc analysis or Dunnett's multiple comparison test, respectively. The analyses were performed with SAS version 9.4 (SAS Institute).

RESULTS

Baseline characteristics of the groups and biomarkers of fibrinolysis and coagulation are shown in **Table 1**. The mean ± SD baseline Lp(a) levels in the placebo and IONIS-APO(a)$_{Rx}$ groups were 362.1 ± 89.9 nmol/l and 477.3 ± 55.9 nmol/l, respectively. The mean size of apo(a) isoforms was 16–17, and *LPA* SNPs r10455872 or rs3798820, which are associated with elevated Lp(a), were present in >80% of patients. The mean ± SD change in Lp(a) was −69.3 ± 12.2% versus −5.4 ± 6.9% ($P < 0.0010$) at day 85/92/99 and −15.6 ± 8.9% versus 3.2 ± 12.2% ($P = 0.003$) at day 190 in the IONIS-APO(a)$_{Rx}$ versus placebo, respectively. The IONIS-APO(a)-L$_{Rx}$ group had baseline Lp(a) levels of 194.0 ± 52.8 nmol/l.

Despite the highly elevated baseline Lp(a) levels and the significant reduction in Lp(a), the mean percentage change (**Fig. 2**) or absolute change (supplemental Fig. S1, supplemental appendix) in clot lysis times in the treatment group or placebo group showed no significant differences or any significant temporal changes. There were also no significant changes in the biomarkers of fibrinolysis and coagulation and no significant correlations between these biomarkers and clot lysis time at any time point (**Table 2**).

Substantial interindividual differences in clot lysis times were observed in the cohorts (supplemental Fig. S2, supplemental appendix). Accordingly, we also measured lysis times in a group of 16 healthy adult males with a mean ± SD age of 41.2 ± 11.7 years and range of Lp(a) concentrations [median (interquartile range): 53.5 (72.5) nmol/l]. A similar range and distribution of lysis times were observed in this cohort (supplemental Fig. S2, supplemental appendix), and no correlation between Lp(a) concentration and clot lysis time was observed (**Fig. 3**).

In a series of control experiments, ex vivo clot lysis assays were performed using plasma from a single donor with low Lp(a) levels (12.5 nmol/l) supplemented with increasing concentrations of purified Lp(a) [single 16K apo(a) isoform] or a 17K form of recombinant apo(a). The addition of Lp(a) up to 200 nmol/l had no effect on clot lysis (**Fig. 4**). Similar findings were obtained using purified Lp(a) from donors with either a single 18K or double 26/28K isoforms (data not shown). In contrast, the addition of the recombinant 17K apo(a) dose-dependently increased lysis time (Fig. 4).

To examine the involvement of different domains of apo(a) in the inhibition of fibrinolysis, we used a series of recombinant variants (**Fig. 5**). Deletion of the strong lysine binding site in the KIV_{10} in the context of the 17K variant had no impact on its antifibrinolytic effect. Interestingly, variants smaller than 17K exhibited progressively smaller antifibrinolytic effects as they became shorter; the 6K variant, which lacks $KIV_{1–4}$ and is not observed physiologically, had no antifibrinolytic effect (Fig. 5).

DISCUSSION

This study demonstrates that potent reductions in Lp(a) in patients with elevated Lp(a) did not result in significant changes in ex vivo clot lysis or in biomarkers of coagulation and fibrinolysis. Importantly, these patients' Lp(a) represented levels at approximately the 98–99th percentile of population levels, which are approximately >175 mg/dl or >437 nmol/l (19, 25). Additionally, they had very small isoforms, and most had *LPA* SNPs associated with elevated Lp(a). Therefore, the patients in this study would be the most likely subset of patients with elevated Lp(a) to have baseline antifibrinolytic effects.

These data are consistent with most recent studies showing no association of elevated Lp(a) with deep venous thrombosis (13, 14), which represents a more pure form of thrombosis than myocardial infarction or ischemic stroke where atherosclerosis is concomitantly present. Lp(a) may not exert a major effect on thrombosis in vivo, and its association with myocardial infarction may be attributable to effects on inflammation, oxidation, and atherosclerosis as primary mechanisms (2).

It is notable that a substantial interindividual variability is observed in clot lysis time, both in our subjects with very high Lp(a) levels as well as a sample of healthy adults representing a broad range of baseline Lp(a) concentrations. In agreement with the lack of impact of Lp(a) lowering on

TABLE 1. Baseline characteristics and coagulation/fibrinolysis parameters

Characteristic	Placebo ($n = 10$)	IONIS-APO(a)$_{Rx}$ ($n = 7$)	P	IONIS-APO(a)-L$_{Rx}$ ($n = 3$)
Age (years)	57 ± 10	60 ± 9	0.440	58 ± 9
Gender (M:F)	6:4	2:5	0.335	1:2
BMI (kg/m^2)	28.4 ± 3.7	28.9 ± 5.4	0.835	22.4 ± 1.2
Race [n (%)]			1.00	
White	9 (90.0)	7 (100.0)		2 (66.7)
Black	1 (10.0)	0 (0.0)		1 (33.3)
Medical history				
Hypertension	4 (40.0)	2 (28.6)	1.000	0 (0.0)
Diabetes mellitus	0 (0.0)	1 (14.3)	0.412	0 (0.0)
Coronary artery disease	4 (40.0)	1 (14.3)	0.338	0 (0.0)
Myocardial infarction	4 (40.0)	0 (0.0)	0.103	0 (0.0)
Percutaneous coronary intervention or coronary-artery bypass grafting	2 (20.0)	1 (14.3)	1.000	0 (0.0)
Stroke or transient ischemic attack	0 (0.0)	0 (0.0)	NA	0 (0.0)
Carotid or peripheral artery disease	0 (0.0)	1 (14.3)	0.412	0 (0.0)
Aortic stenosis	0 (0.0)	0 (0.0)	NA	0 (0.0)
Lipid-lowering medications				
Statin	8 (80.0)	5 (71.4)		0 (0.0)
Ezetimibe	4 (40.0)	3 (42.9)		0 (0.0)
Other	0 (0.0)	0 (0.0)		0 (0.0)
Any lipid-modulating medication	8 (80.0)	5 (71.4)	1.000	0 (0.0)
Lp(a) (nmol/l)				
Baseline	362.1 ± 89.9	477.3 ± 55.9	0.009	194.0 ± 52.8
End of treatment	341.9 ± 87.2	148.0.3 ± 64.7	<0.001	12.1 ± 4.9
End of follow-up	368.3 ± 73.5	401.2 ± 50.1	0.322	54.8 (44.7)
Lp(a) size, major isoform (number of kringle repeats)	16.7 ± 1.5	16.7 ± 0.8	0.917	17.3 ± 1.5
LPA snp group [n, %]				
rs3798220 homozygotes	1 (10.0)	0 (0.0)		0 (0.0)
rs10455872 homozygotes	1 (10.0)	1 (14.3)		0 (0.0)
rs3798220 and rs10455872 heterozygotes	2 (20.0)	2 (28.6)		0 (0.0)
heterozygous rs10455872	2 (20.0)	1 (14.3)		1 (33.3)
heterozygous rs3798220	3 (30.0)	2 (28.6)		1 (33.3)
Either rs3798220 or rs10455872	9 (90.0)	6 (83.7)	1.000	2 (66.7)
Estimated Lp(a)-C (mg/dl)	45.3 ± 11.2	59.7 ± 7.0	0.009	24.3 ± 6.6
LDL-C (mg/dl)	109.7 ± 36.8	116.5 ± 39.5	0.773	139.0 ± 35.1
Corrected LDL-C	64.4 ± 41.1	56.8 ± 40.2	0.962	114.7 ± 41.1
apoB (mg/dl)	90.5 ± 18.6	96.9 ± 23.2	0.535	95.2 ± 18.1
Total cholesterol (mg/dl)	190.1 ± 51.4	192.6 ± 39.4	0.914	217.3 ± 44.2
HDL-C (mg/dl)	55.5 ± 28.4	51.4 ± 9.7	0.721	61.2 ± 17.8
Triglycerides (mg/dl)	125.6 ± 38.2	123.3 ± 26.3	0.892	85.5 ± 20.3
OxPL-plasminogen (nM)	160.6 ± 88.7	134.3 ± 39.1	0.810	102.8 ± 82.3
OxPL-apoB (nM)	27.2 ± 4.4	32.7 ± 3.0	0.012	24.8 ± 7.0
OxPL-apo(a) (nM)	57.5 ± 13.9	63.9 ± 11.3	0.337	62.7 ± 26.1
Plasminogen (mg/dl)	7.6 ± 4.4	7.1 ± 2.1	0.799	8.8 ± 3.4
Factor XI antigen (IU/ml)	1.25 ± 0.25	1.45 ± 0.25	0.111	
Fibrinogen (mg/ml)	2.47 ± 0.88	3.01 ± 0.83	0.219	
PAI-1 antigen (pg/ml)	2077 ± 1686	1732 ± 1022	0.638	
TAFI antigen (μg/ml)	5.29 ± 1.55	5.23 ± 2.27	0.949	

P values compare placebo to IONIS-APO(a)$_{Rx}$. End of treatment: day 85/92/99 for IONIS-APO(a)$_{Rx}$ and day 36 for IONIS-APO(a)-L$_{Rx}$; end of follow up: day 190 for IONIS-APO(a)$_{Rx}$ and day 113 for IONIS-APO(a)-L$_{Rx}$. For the continuous data, the comparisons between the two treatment arms [IONIS-APO(a)$_{Rx}$ treatment and placebo] in the IONIS-APO(a)$_{Rx}$ trial were performed using the two-sample *t*-test or Wilcoxon rank-sum test. For the categorical data, the comparisons between the two treatment arms in the IONIS-APO(a)$_{Rx}$ trial were performed using Fisher's exact test.

lysis times, there was no correlation between baseline Lp(a) levels and clot lysis time.

Allowing for the fact that the current ex vivo assays may not ideally reflect in vivo conditions, it is possible that prothrombotic effects of Lp(a) in vivo are present beyond the inhibition of fibrinolysis and that are not captured by this assay; some previously documented examples include the promotion of platelet activation (26) and inhibition of TFPI (27). In addition, contradictory data have been reported on the relationship between rs3798220 carrier status and clot lysis times ex vivo (28, 29). In general, the stoichiometry does not favor in vivo inhibition of plasminogen activity by Lp(a) because the plasma levels of plasminogen (5–20 mg/dl or 543–2,174 nmol/l) are usually in excess to Lp(a) even with the highest levels of Lp(a) at the 99th percentile of levels (437 nmol/l). However, antifibrinolytic effects of Lp(a) may be manifest in local microenvironments in which excessive accumulation of Lp(a) may prevent optimal plasminogen activation to plasmin to effect fibrinolysis in preexisting thrombi. Furthermore, Lp(a) may be synergistic with other prothrombotic risk factors and may not have a significant effect on its own (16).

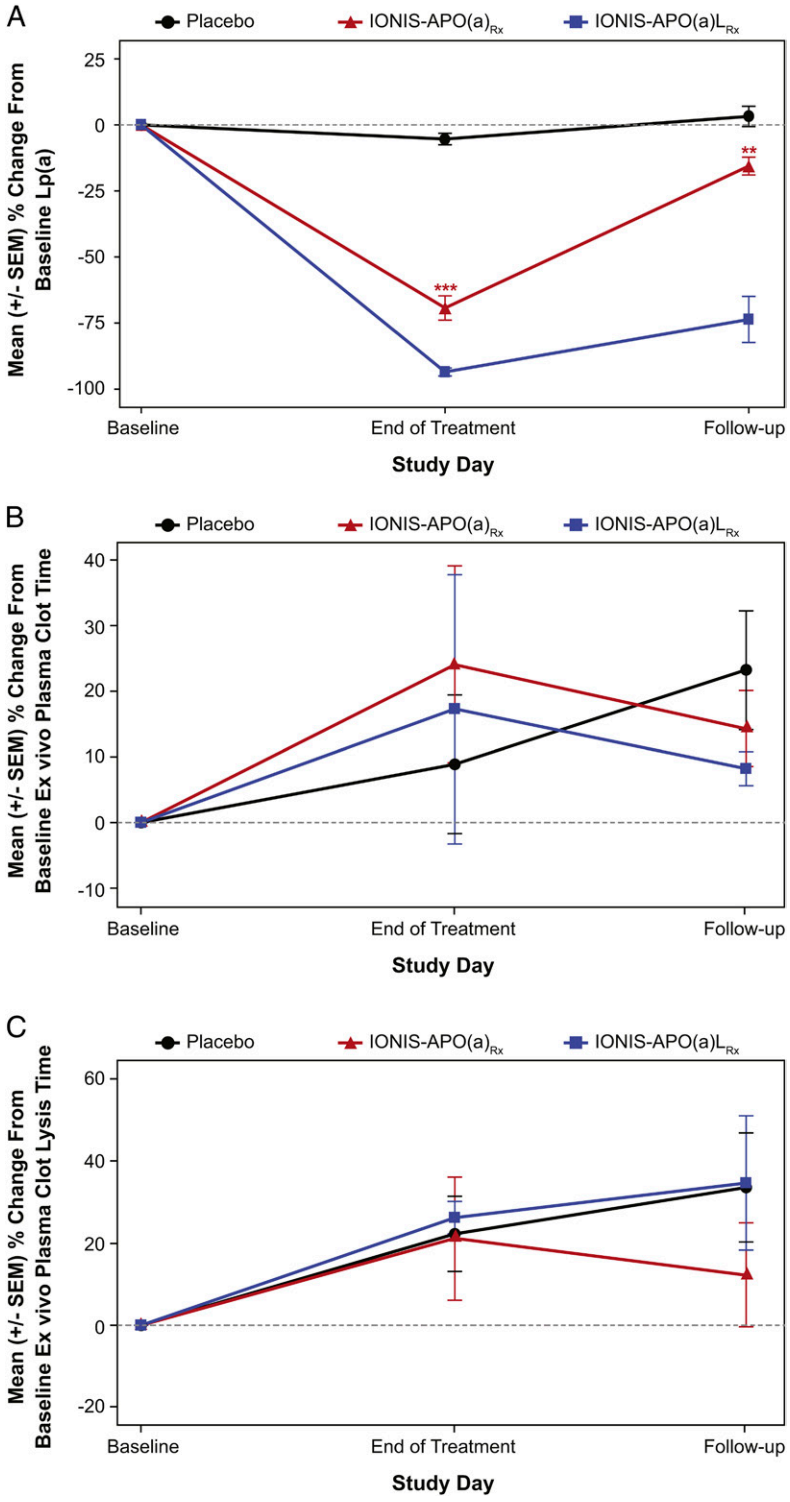

Fig. 2. Ex vivo clot lysis times comparing placebo to IONIS-APO(a)$_{Rx}$ and IONIS-APO(a)-L$_{Rx}$. The mean percentage change from baseline in Lp(a), clot time, and clot lysis time are shown in panels A, B, and C, respectively. ***$P \leq 0.001$, **$P \leq 0.01$, and *$P \leq 0.05$.

The lack of effect of purified Lp(a) on clot lysis is surprising in light of an extensive body of evidence that apo(a) itself inhibits fibrinolysis and plasminogen activation (6). Only one study utilized our approach of adding Lp(a) to plasma and measuring tPA-mediated clot lysis, and the authors found that ~45 nmol/l Lp(a) reduced the extent of clot lysis by at most 25%, although the method used for preparing the clots and monitoring clot lysis was different from the current method (7). Several studies have detected correlations between Lp(a) levels and the inhibition of plasma clot lysis in vitro with Lp(a)-lowering hormone replacement therapy (30) or in the setting of disorders such as residual vein obstruction and cerebral venous sinus thrombosis (31), or myocardial infarction (32). In another study, it was found that patients with type 2 diabetes mellitus had longer clot lysis times than healthy control subjects, although no difference in lysis time by quartile of Lp(a) levels was found in either group (33). Our study has an

TABLE 2. Effect of antisense therapy on coagulation/fibrinolysis biomarkers in patients treated with placebo or IONIS-APO(a)R_x

Characteristic	Baseline			End of Treatment			End of Follow-Up		
	Placebo	IONIS-APO(a)$_{Rx}$	*P*	Placebo (% change)	IONIS-APO(a)$_{Rx}$ (% change)	*P*	Placebo (% change)	IONIS-APO(a)$_{Rx}$ (% change)	*P*
Lp(a) (nmol/l)	362.1 ± 89.9	477.3 ± 55.9	0.009	341.9 ± 87.2 (−5.4 ± 6.9)	148.0 ± 64.7 (−69.3 ± 12.2)	<0.001	368.3 ± 73.5 (3.2 ± 12.2)	401.2 ± 50.1 (−15.6 ± 8.9)	0.003
Plasminogen (mg/dl)	7.6 ± 4.4	7.1 ± 2.1	0.799	7.9 ± 4.0 (14.8 ± 63.8)	6.1 ± 2.1 (−5.7 ± 47.0)	0.482	7.1 ± 3.2 (22.1 ± 83.4)	6.5 ± 3.4 (3.0 ± 63.0)	0.810
Factor XI antigen (IU/ml)	1.25 ± 0.25	1.45 ± 0.25	0.111	1.25 ± 0.19 (1.7 ± 13.8)	1.33 ± 0.21 (−7.7 ± 7.3)	0.124	1.29 ± 0.25 (4.7 ± 15.5)	1.36 ± 0.27 (−6.7 ± 8.9)	0.102
Fibrinogen (mg/ml)	2.47 ± 0.88	3.01 ± 0.83	0.219	2.59 ± 0.86 (12.7 ± 43.2)	2.46 ± 0.48 (−16.5 ± 11.5)	0.104	2.26 ± 0.78 (−3.3 ± 30.7)	2.86 ± 0.68 (−0.2 ± 30.4)	0.840
PAI-1 antigen (pg/ml)	2,077 ± 1,686	1,732 ± 1,022	0.638	3,242 ± 2,442 (65.0 ± 95.7)	1,679 ± 1,544 (−5.2 ± 43.1)	0.075	2,543 ± 1,837 (44.7 ± 91.2)	2,081 ± 1,107 (54.7 ± 87.2)	0.737
TAFI antigen (ug/ml)	5.29 ± 1.55	5.23 ± 2.27	0.949	5.60 ± 1.44 (8.5 ± 19.7)	5.14 ± 2.66 (−2.7 ± 19.4)	0.266	5.70 ± 1.16 (12.4 ± 26.7)	5.83 ± 2.24 (14.9 ± 23.6)	0.475

P values compare placebo to IONIS-APO(a)$_{Rx}$. End of treatment: day 85/92/99; end of follow-up: day 190. The comparisons between IONIS-APO(a)$_{Rx}$ treatment, and placebo were performed using the two-sample *t* test or Wilcoxon rank-sum test.

Fig. 3. Ex vivo clot lysis in healthy adults representing a range of Lp(a) concentrations. Baseline plasma samples for healthy adults (n = 16) enrolled in a phase 1 study of IONIS-APO(a)$_{Rx}$ were used. Lysis times are plotted against baseline Lp(a) concentration; no significant correlation was observed.

advantage over these in that we used individual subjects receiving either placebo or IONIS-APO(a)$_{Rx}$ as their own control, minimizing selection bias and other confounders.

In contrast to Lp(a), the human apo(a) isoform 17K had a clear antifibrinolytic effect in our assay, in agreement with previous findings from both in vitro and in animal models (10–12). These data suggest that, compared with Lp(a), free apo(a) is a more potent antifibrinolytic agent; this may explain some of the findings in the literature with such constructs (6). However, the clinical significance of

Fig. 4. Effect of purified Lp(a) or apo(a) on ex vivo plasma clot lysis. Plasma from an individual with low Lp(a) was supplemented with purified Lp(a) or 17K recombinant apo(a). Mixtures were combined with thromboplastin to induce clot formation and tPA to induce subsequent clot lysis. Clot lysis times were evaluated from measurements of turbidity over time at 37°C. Clot lysis assays were carried out in a manner similar to the patient samples in Fig. 2, except that tPA concentration was doubled to account for the antifibrinolytic effect of 17K. The data shown are the means ± SDs of four independent experiments. By ANOVA with Tukey's post hoc analysis, asterisks indicate significant differences between Lp(a) and apo(a). $^{**}P < 0.01$ and $^{***}P < 0.001$.

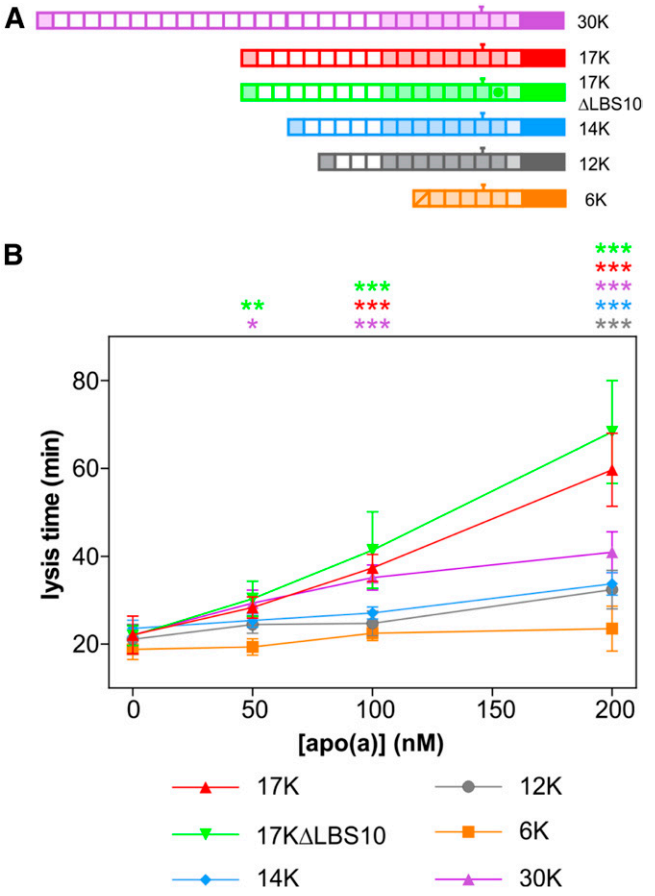

Fig. 5. Antifibrinolytic effect of recombinant apo(a) variants. A: Topology of the variants. The circle represents the mutation in KIV_{10} abolishing the strong lysine binding site. The bar indicates the unpaired cysteine in KIV_9. KIV_2 repeats are unshaded; KIV_1 and $KIV_{3–10}$ are lightly shaded, and KV is very lightly shaded. The protease-like domain is darkly shaded. B: Ex vivo clot lysis assays were carried as described for Fig. 4. The data shown are the means ± SDs of four independent experiments. By ANOVA with Dunnett's multiple comparison tests, asterisks (color-coded by variant) indicate significant differences for the respective variants compared with the absence of apo(a). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

this is not clear, as very little (<5%) free apo(a) exists in human plasma of untreated patients, except perhaps in renal failure (34–36). In the current era of achieving very low plasma apoB-100 levels (37), it is conceivable that inadequate amounts of apoB-100 will be available for Lp(a) particles to be generated and that increased amounts of free apo(a) may circulate, a paradigm shown previously in Lp(a)-transgenic mice (38) and that we have postulated may occur in patients (39).

One potential reason why apo(a) may not inhibit fibrinolysis in the context of the Lp(a) particle is that the kringle domains involved are blocked by association with apoB-100. We do not believe that this is likely because the recombinant apo(a) would rapidly associate with the excess LDL present in the plasma samples. We also performed experiments in which we added purified LDL up to 400 nmol/l (particle concentration) to the assays, but this did not blunt the antifibrinolytic effect of 17K (data not shown).

Our experiments with recombinant variants of apo(a) also yielded some unexpected findings. First, the strong lysine binding site in KIV_{10} [the kringle that also harbors the covalently bound oxidized phospholipid in apo(a) (3)] is dispensable for inhibiting fibrinolysis. This differs from our previous findings that ablation of this site lowers the ability of apo(a) to inhibit tPA-mediated plasminogen activation and to attenuate plasmin-mediated conversion of Glu-plasminogen to Lys-plasminogen (24, 40). We also found that smaller apo(a) variants, including rare but clinically observable 14K and 12K variants, display a drastically reduced antifibrinolytic potency. The 6K variant, which potently inhibits tPA-mediated plasminogen activation and Lys-plasminogen formation, had no antifibrinolytic effect at all (24, 40, 41). These results indicate that fragments of apo(a) generated by proteolysis of Lp(a) in vivo would not generate species capable of inhibiting fibrinolysis.

Limitations of this study include the small study size, and thus these data need to be confirmed in larger studies. Furthermore, ex vivo clot lysis assays may not necessarily reflect in vivo properties of Lp(a) on fibrinolysis. Finally, the variation in the clot lysis times for a given patient observed at the different time points may have obscured any effect of Lp(a) lowering.

In conclusion, in patients with highly elevated Lp(a), we find no evidence that a very potent reduction in Lp(a) affects ex vivo fibrinolysis. These observations suggest the main pathophysiological contribution of Lp(a) to CVD and aortic stenosis may be through its proatherogenic and proinflammatory components, such as oxidized phospholipids (42). Further investigation is required using larger cohorts and additional methodologies to confirm these findings.

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