Title: Impact of LDL-cholesterol lowering on platelet activation

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Introduction

Hyperlipidemia as exemplified by familial hypercholesterolemia is associated with increased platelet activation and an underlying pro-coagulant state[1-3]. Hyperlipidemia primes platelets and increases platelet activation in response to various agonists[1, 4]. Plasma cholesterol levels appear to have a critical role in modulating platelet activity as hypercholesterolemia increases platelet activation more potently than hypertriglyceridemia[1, 4]. Plasma levels of platelet activation markers such as thrombin-antithrombin complex (TAT), soluble P-selectin (sP-selectin), soluble CD40L (sCD40L) or P-selectin exposure at surface of platelets are increased in hypercholesterolemic patients[5-7]. Increased levels of the platelet activation markers are associated with increased platelet membrane cholesterol content in hypercholesterolemia[8]. Hyperlipidemia increases platelet activation likely via multiple mechanisms[9, 10]. Oxidized LDL or oxidized phospholipids, which are increased in hyperlipidemia[9, 11], serve as ligands of platelet CD36 and activate platelets[9, 12]. *In vitro* cholesterol-loading increases [13] while cholesterol-unloading decreases human platelet activation.[14, 15] Recently, we showed that cholesterol enrichment of platelets increased platelet activation via a mechanism involving ↓LYN Kinase activation→↓SHIP-1 activity→↑PIP3→↑AKT activity[15].

Statins may show antithrombotic properties[16]. The PROVE-IT trial, a study to assess the impact of statins on acute coronary syndrome (ACS), demonstrated an early beneficial effect after 30 days of follow-up that significantly reduced the risk of death, MI or rehospitalization[17]. A retrospective study of 1616 patients (PRISM) also showed significant reduction of the risk during 30 day follow-up in ACS patients with statin therapy[18]. Additional human studies suggest that the benefits may come partly from the antithrombotic properties of statins[16]. Statins reduced the level of TAT, sP-selectin, sCD40L and P-selectin exposure at surface of platelets[8, 19-21]. Reduced levels of P-selectin at the platelet surface were associated with reduced platelet cholesterol content in subjects treated with statins[8] . These studies suggest an early direct effect of statins on platelets and a late prolonged effect that is associated with progressive reduction of plasma LDL-cholesterol levels[16, 22]. Both the early and late effects are proposed to lower platelet activation and contribute to the reduced risk. Consistent with the idea that cholesterol enrichment increases platelet activation, infusions of a reconstituted HDL (rHDL) preparation

reduced *ex vivo* platelet activation in diabetic subjects, likely by promoting cholesterol efflux from platelets[14].

Human genome wide association studies have revealed novel genetic loci associated with coronary heart disease. One such locus resides in *LNK/SH2B3* which in mice is expressed in hematopoietic cells and suppresses thrombopoietin signaling via its receptor MPL. Using human cord blood, we recently showed that the common TT risk genotype (R262W) of *LNK* is associated with expansion of hematopoietic stem cells and enhanced megakaryopoiesis, demonstrating reduced LNK function and increased MPL signaling. To model the human reduced LNK function associated with the TT risk genotype, we used $Lnk^{-/-}$ mice. In mice, hematopoietic *Lnk* deficiency led to accelerated arterial thrombosis, but only in the setting of hypercholesterolemia. In platelets LNK deficiency increased MPL signaling, while cholesterol loading decreased SHIP-1 activation, acting convergently to increase AKT and platelet activation. Increased platelet activation combines with hypercholesterolemia induced myelopoiesis to promote pro-thrombotic platelet/leukocyte aggregate formation and accelerated atherogenesis. Atherosclerotic lesions were characterized by larger size, increased necrotic cores and increased leukocyte-derived DNA nets.

These findings suggest the possibility that in humans, the common LNK variant rs3184504 impacts platelet activation in a fashion dependent on plasma cholesterol levels. In light of these findings, we propose the following studies as an investigator initiated study. The primary goal is to assess the impact of Evolocumab therapy on platelet function of FH patients in a randomized, double blind study. The secondary goal is to determine if platelet activation or the response to Evolocumab therapy is modified by rs3184504 polymorphism. We believe that these investigations will complement ongoing studies to demonstrate that Evolocumab reduces athero-thrombotic risk and aid the decision-making as to whether Evolocumab can reduce the atherothrombotic risk in ACS patients.

Proposed studies.

A. Sample collection: All adult male and female subjects who have a clinical diagnosis of familial hypercholesterolemia (FH) and who are referred to Dr. Ginsberg's Lipid Practice for treatment with PCSK9 inhibitor, with LDL cholesterol levels ≥70 mg/dl on baseline

treatment with statins and/or ezetimibe or intolerant to statin and/or ezetimibe. The platelet counts in peripheral blood should be ≥100,000 per microliter. The subjects that meet these criteria will be recruited for the study.

Blood will be collected as shown in Fig. 1 in the morning from the resting and fasting subjects. An aliquot will be collected for genomic DNA and platelet-rich plasma (PRP) preparation.

Fig. 1 Flow chart of blood sample collection

Step	Procedure
1	Venipuncture with G21 butterfly needle.
2	Collect 2 ml blood into sodium citrate tube as waste tube
3	Collect 3×2 mL blood into 3 Greiner Vacuette tubes containing 3.2% sodium citrate. For VerifyNow assays.
4	Collect 2×3 ml blood into 2 Greiner Vacuette tubes containing 3.2% sodium citrate. For platelet activation assays.
5	Collect 1×2 ml blood into Vacuette tube containing EDTA. For CBC assay.
6	Collect 2×2 ml blood for metabolic panel, lipid panel and TSH assays.
7	Collect urine for urinalysis.
8	Pregnancy test with stick assay.

B. Power Calculation:

The effect of PCSK9 inhibitors, including evolocumab, on platelet activation measures has not been assessed previously. However, our hypothesis is based on the LDL-cholesterol lowering effect of evolocumab, and evolocumab has been demonstrated to have at least as potent an effect on LDL lowering as statins[23, 24]. Statin therapy in hypercholesterolemia patients results in 50% reduction in LDL-cholesterol levels and add-on evolocumab therapy indicated an additional 50-60% reduction[23-25]. Therefore, we base our power estimates on platelet activation data available for statins.

Primary measure/platelet aggregation - The primary measurement will be ADP-stimulated platelet aggregation as assessed by the commercially-available VerifyNow P2Y12 assay (Accriva Diagnostics). The assay read-outs are 1) PRU, platelet reactivity units (specific for P2Y12 receptors); 2) BASE, total platelet function despite P2Y12 receptor blockage; and 3) IPA, percent platelet P2Y12 inhibition calculated as [(BASE - PRU) / BASE] X 100. Two reports involving patients on antiplatelet therapy with high residual platelet reactivity demonstrated an effect of statins on lowering platelet aggregation via the VerifyNow assay. In CAD patients treated with clopidogrel, the addition of atorvastatin for 30 days resulted in an absolute mean difference of 102 ± 54 PRU compared to baseline, corresponding to a 37 ± 14% IPA[25]. In a separate study of CAD/diabetic patients treated with clopidogrel, the addition of atorvastatin for 30 days resulted in an absolute mean difference of 106 ± 75 PRU compared to baseline, corresponding to a 33 ± 20% IPA[8]. Similar effect sizes and variabilities of statins on reductions in platelet aggregation were observed in type II

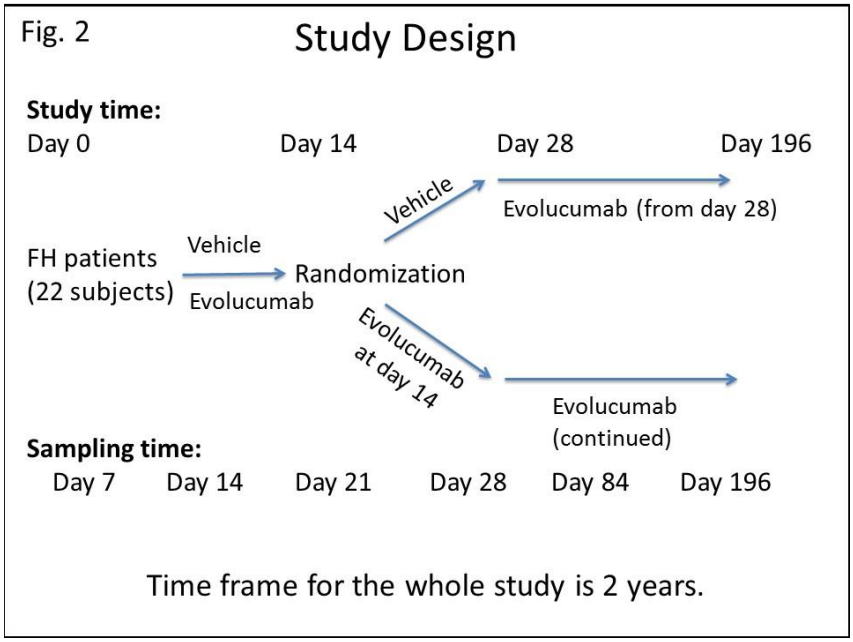
hyperlipidemic patients using light transmittance aggregometry[21]. The relative reductions were reported as 30 ± 23% (pravastatin), 19 ± 16% (atorvastatin), and 22 ± 22% (simvastatin)[7].

The reference range for the VerifyNow assay in individuals not on antiplatelet therapy is 180-376 PRU. These data are continuous and normally distributed[5-6]. Thus, means and standard deviations will be used to describe reductions in platelet aggregation. Based on reported literature indicating additional effects of statins on platelet function following 30-day vs shorter periods of treatment, we have revised our experimental design to have a primary endpoint of 30-day treatment[26,21]. Within-group thirty day evolocumab treatment will be tested by one-group paired t-test (two-sided) using each subject as its own control (evolocumab treatment vs vehicle pre-treatment control). Between-group thirty day evolocumab treatment will be tested by standard two-group t-test (two-sided, evolocumab vs vehicle control group run in parallel). The sample size estimates have been run using one-group, paired t-test published data. Using PRU mean difference = 102[26], SD = 54[26], power = 0.8 and alpha = 0.05, we estimate 6 subjects. Since the effects of statins on platelet aggregation may be mediated by both LDL-lowering and non-LDL-lowering mechanisms, we can calculate the sample size based on 50% reduced mean difference (PRU = 51). Then, we estimate 11 subjects. An equal number of subjects will be required for the placebo group run in parallel. Target enrollment will include all patients screened; we estimate that 10% may not be eligible based on exclusion criteria and/or may not return for scheduled blood draws. Thus, for 80% power, the target enrollment is 24 subjects over two years.

Secondary measures – Secondary measures will include various cellular and circulating markers of platelet activation measured by flow cytometry or commercially-available enzyme-linked immunoassays (ELISAs). The target enrollment of 24 subjects should provide at least 80% power to detect differences between treatment groups based on data reported in the literature. For example, platelet P-selectin level measured by flow cytometry and reported as % positive cells (out of 50,000 cells counted) showed a mean reduction ± SD of 10.9 ± 6.0 (simvastatin), 10.9 ± 5.6 (atorvastatin), 7.2 ± 6.8 (fluvastatin) and 9.0 ± 6.8 (pravastatin) following 30 days of statin treatment in hypercholesterolemic patients compared with untreated controls[27]. In a separate study of stroke patients with hypercholesterolemia, platelet P-selectin showed a mean reduction of 41 ± 27% positive cells in response to thrombin following 6 months of treatment with simvastatin compared to baseline measurements in the same subjects before statin treatment[28]. An additional

effect of simvastatin treatment was observed for soluble P-selectin serum concentrations measured by ELISA. Soluble P-selectin showed a mean reduction of 46.4 ± 49 ng/ml compared to baseline measurements in the same subjects before statin treatment[28]. Other secondary measures include VerifyNow IIb/IIIa and VerifyNow Aspirin assays.

C. Study Design: Amgen will provide Evolocumab and placebo as open label. An independent biostatistician will generate a randomization table and provide it to Research Pharmacy of CUMC and Research Pharmacy will randomize the subjects accordingly. All patients recruited will start with placebo and blood and urine samples will be collected at day 7 and day 14. Then, the subjects will be randomized so that half will continue to receive placebo and half will receive Evolocumab via subcutaneous injection of 140 mg every 14 days. Blood and urine samples will be collected at day 21 and day 28. Then, all patients will receive Evolocumab 140 mg every 14 days and the treatment will continue. Blood and urine samples will be collected following Evolocumab therapy at day 84 and 196 (Fig. 2). While every effort will be made to collect the blood and urine samples on the indicated time points, variation for the sample collection within 1-3 days at the indicated time point will be allowed. The power of this design is that we can compare any changes due to Evolocumab therapy relative to a placebo control.



Based on the power calculations as detailed above, recruitment of total 20 participants into this study will give ~80% power for statistical significance of 0.05. We target to recruit 24 subjects over 2 year period. However, we anticipate that some participants will drop out from the study and incomplete data will be obtained. We will continue to recruit the subjects until the complete data sets for 22 subjects are obtained. Incomplete data from the dropout may not be used unless data up to 28 days at least are collected.

D. Genotyping: rs3184504 genotyping will be performed using the genomic DNA samples

and rs3184504 specific PCR genotyping primers from Invitrogen.

E. Assays: We propose to use a clinically validated system, VerifyNow, as the assay for assessment of platelet activation state. Compared with other assays, this system requires ~2 ml whole blood, with minimal ex vivo manipulations, minimal hands-on time and no pipetting or sample preparation. It is fast (10 min for result presentation) and approved as a clinical lab test of platelet reactivity. Thus, the results obtained this way should be more reliable when the data need to be compared with results from other studies using the same assay. In principle, this assay is designed to measure platelet function based upon the ability of activated platelets to bind fibrinogen. Fibrinogen-coated microparticles aggregate in whole blood in proportion to the number of unblocked platelet GP IIb/IIIa receptors. Light transmittance increases as activated platelets bind and aggregate fibrinogen-coated beads. The instrument measures this change in optical signal caused by aggregation. We propose to use VerifyNow IIb/IIIa, VerifyNow PRUTest, and VerifyNow Aspirin assays, testing platelet responses to thrombin-like peptide, ADP and arachidonic acid, respectively. In addition to the VerifyNow assay, basal level of P-selectin and active form of GPIIbIIIa at surface of platelets will be determined by flow cytometry. In order to reduce the variation generated by manipulation of blood samples in vitro, we will use 1% paraformaldehyde to fix an aliquot of the whole blood sample and basal levels of P-selectin and active GPIIbIIIa at surface of platelets will be determined by flow cytometry after staining with specific P-selectin or PAC-1 antibody (PAC-1 specific for active GPIIbIIIa)[29, 30]. Plasma levels of TAT, sP-selectin and sCD40L will be determined by ELISA assays. Levels of plasma LDL cholesterol, HDL cholesterol, triglyceride and platelet total cholesterol content will be determined. All these assays are validated assays.

F. Analysis: The primary hypothesis is that Evolocumab will reduce thrombin-peptide stimulated platelet activation as determine by VerifyNow. Secondary hypothesis is related to basal and ADP or arachidonic acid stimulated activation, direct measurements of platelet activation by flow cytometry or levels of the surrogate markers. This will help assess the validity of these markers for larger ongoing studies that we are performing in collaboration with Amgen using EDTA plasma samples obtained in phase 2 development studies of Evolocumab. Even though the statistical power is limited by small sample size, the potential modification of these phenotypes by rs3184504 polymorphism will be examined.

Time Frame: two years

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