Impact of the PCSK9 Inhibitor Evolocumab on the Pharmacodynamic Effects of Clopidogrel in Patients with Atherosclerotic Cardiovascular Disease and High or Normal On-Treatment Platelet Reactivity

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Background and Significance

Dual antiplatelet therapy (DAPT) with aspirin and a $P2Y_{12}$ receptor inhibitor represents the standard of care for the long-term secondary prevention of atherothrombotic events in patients with acute coronary syndrome (ACS) or undergoing percutaneous coronary intervention (PCI) [1]. Moreover antiplatelet therapy is commonly used in patients with other atherothrombotic disease manifestations, such as stroke and peripheral arterial disease (PAD). Clopidogrel is the most widely used $P2Y_{12}$ receptor inhibitor and is the only agent of this class currently recommended in patients with stable coronary artery disease (CAD) undergoing PCI, and for the treatment of stroke or PAD [2]. Although the efficacy of DAPT with aspirin and clopidogrel has been consistently shown in different clinical settings, rates of ischemic recurrences remain elevated despite this treatment regimen, especially in high risk patients [1,3]. This has been in part attributed to the high interindividual variability in responses to clopidogrel [3,4]. Clopidogrel is a pro-drug which requires a two-step metabolic transformation to exert its antiplatelet effect. Pharmacodynamic (PD) studies have shown that approximately 30-40% of patients experience high on-treatment platelet reactivity (HPR) while receiving clopidogrel treatment [3,4]. Importantly HPR status has been strongly associated with an increased risk of ischemic events, in particular stent thrombosis, in patients with ACS and following PCI [4]. This underscores the need for strategies aimed to reduce HPR rates in patients treated with clopidogrel. Several factors (clinical, cellular, and genetic) have been associated with HPR on clopidogrel [3,4]. Among these, genetic polymorphisms of the hepatic cytochrome P450 (CYP) 2C19 gene, the enzyme involved in both metabolic steps of transformation of clopidogrel pro-drug into its active metabolite, have shown to affect clopidogrel PD and pharmacokinetic and impact clinical outcomes [3,4]. In particular, the **2* and **3* loss-of-function

(LOF) allelic variants confer loss of enzyme function, which leads to attenuated clopidogrel bioactivation, lower platelet inhibition and worse outcomes [3,4].

Multiple approaches have been advocated to reduce HPR rates. Intuitively, the use of the more potent $P2Y_{12}$ receptor inhibitors prasugrel and ticagrelor represent potential treatment options [1]. However, their increased costs and risk of bleeding complications compared with clopidogrel have limited their broad uptake in clinical practice [1]. The pleiotropic effects associated with lipid lowering therapies, in particular statins, have been subject to extensive research [5]. In a previous study we showed that treatment with high-dose atorvastatin in addition to double-dose clopidogrel reduced platelet reactivity significantly more than double-dose clopidogrel alone in statin-naïve patients with stable CAD and HPR [6]. To date, the exact biological mechanisms involved in the statin modulation of platelet function are not fully understood, although likely attributed to both its lipid-lowering and non-lipid-related effects [7,8]. In fact, there is increasing evidence that circulating lipoproteins affect platelet function. In particular, LDL, VLDL, and especially oxidized LDL are atherogenic lipoproteins and increase platelet activation [9]. Also, familial hypercholesterolaemia is associated with increased platelet activity, such as hyperaggregability, and the application of a lipid-lowering drug resulted in a reduction of platelet reactivity [10].

Evolocumab is a monoclonal antibody targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) that is administered subcutaneously (s.c.) at a dosage of 140 mg every 2 weeks or 420 mg once monthly [11]. In clinical trials in patients with primary hypercholesterolemia or mixed dyslipidemia, evolocumab was more effective than placebo and/or ezetimibe in reducing LDL cholesterol, including when added to statin therapy [11-13]. Importantly, in an exploratory analysis, the use of evolocumab plus standard therapy, as compared with standard therapy alone, significantly reduced the incidence of cardiovascular events [13]. Whether the reduction in cardiovascular events

is simply due to LDL reduction or might be related to other mechanisms is currently subject of investigation. Although LDL reduction with statin therapies has been associated with reduction in platelet reactivity, to date the effects on platelet aggregation of adjunctive lipid lowering with evolocumab has not been explored.

Study aims

The primary aim of the present study is to investigate the effects of evolocumab in addition to statin therapy on platelet reactivity in patients with atherosclerotic cardiovascular disease (ASCVD) while on clopidogrel treatment. Secondary aims will include the effects on cholesterol levels.

Research Plan

Study Population

Inclusion criteria:

1. Patients with ASCVD, defined as prior ACS, history of myocardial infarction, stable or unstable angina, coronary or other arterial revascularization, stroke, transient ischemic attack, or PAD presumed to be of atherosclerotic origin.
2. On therapy with clopidogrel (75mg od), with or without low-dose aspirin (81mg od), as per standard-of-care for at least 30 days.
3. HPR, defined as $P2Y_{12}$ reaction units (PRU) > 208 by VerifyNow P2Y12, or normal platelet reactivity (NPR), defined as PRU between 85 and 208.

4. Fasting LDL-cholesterol ≥70 mg/dL or a non-high-density lipoprotein cholesterol (HDL-C) of ≥100 mg/dL after ≥2 weeks of optimized stable lipid-lowering therapy with maximally tolerated dose of statin, which would ideally include a high-intensity statin, but must be at least moderate intensity statin (i.e. atorvastatin 20 mg or equivalent, with or without ezetimibe. Maximal tolerated dose will be defined based on patient clinical history (no statin re-challenge will be performed).
5. Age ≥ 18 years old.

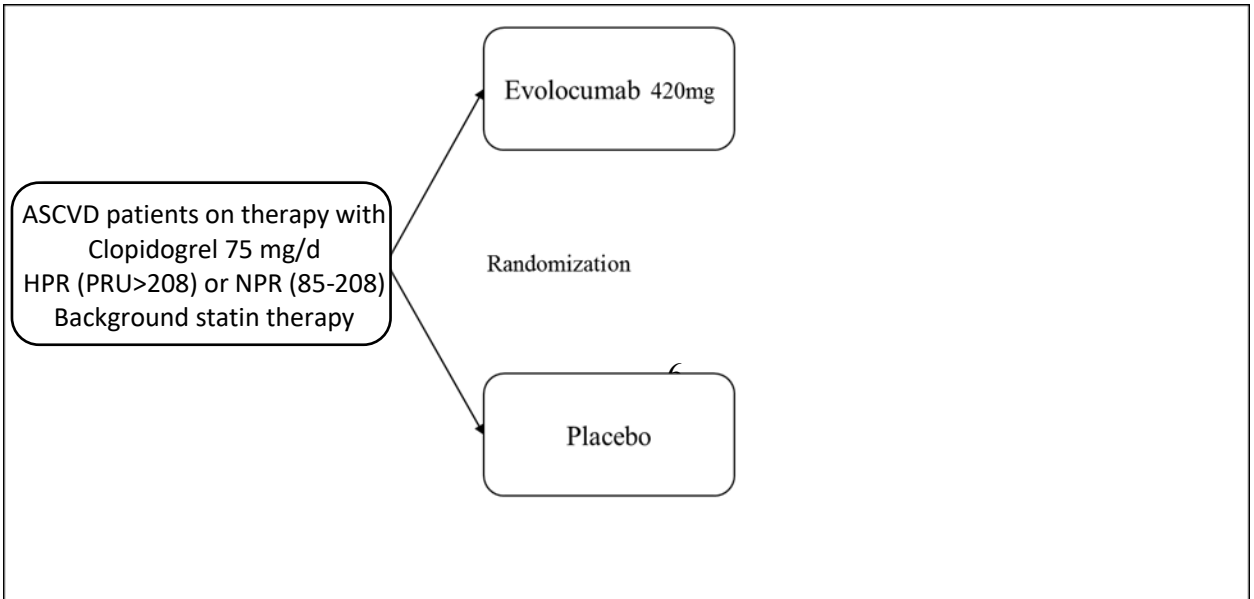
Exclusion criteria:

1. On treatment with any oral anticoagulant (vitamin K antagonists, dabigatran, rivaroxaban, apixaban, edoxaban).
2. On treatment with any antiplatelet agent other than aspirin and clopidogrel in the past 14 days.
3. Use of PCSK9 inhibitors in the past 90 days
4. Creatinine clearance <30 mL/minute.
5. Known severe hepatic impairment.
6. History of a serious hypersensitivity reaction to evolocumab
7. Hemodynamic instability
8. Pregnant and breastfeeding women [women of childbearing age must use reliable birth control (i.e. oral contraceptives) while participating in the study].

Research Design

The proposed investigation will be a prospective, randomized, parallel-design, double-blind, placebo-controlled, PD study conducted in patients with ASCVD and HPR (PRU>208) or NPR (PRU 85-208) on clopidogrel. Patients with low platelet reactivity (PRU<85) will be excluded given that their distribution profile of platelet reactivity would be unlikely to unravel a platelet inhibitory treatment effect of evolucomab. The study will be performed at the University of Florida Health Science Center at UF Health Jacksonville - Division of Cardiology. Patients will be recruited in the Cardiology Clinics of our institution and will be screened by Cardiology Research staff, who will verify that all candidates meet inclusion and exclusion criteria. Results from blood tests performed within the last 90 days will be considered valid for screening purposes. If these are not available, a blood sample will be collected for the screening phase.

After providing written informed consent, patients meeting study entry criteria will be randomly assigned to receive either evolocumab 420 mg s.c. or placebo (0.9% sodium chloride s.c. injection). Blood sampling for PD testing will be conducted at 3 time-points: baseline, and 14±2 days and 30±2 days after randomized drug administration. At each time point, blood will be collected before the morning dose of clopidogrel, in order to measure trough levels of platelet inhibition. During study treatment major adverse cardiac events (death, MI, stroke, and urgent revascularization procedures) and serious adverse events (bleeding and other adverse events) will be collected. A flow diagram of the study design is illustrated below.



Laboratory testing

PD testing

Peripheral venous blood samples will be drawn through a short venous catheter inserted into a forearm vein and collected in citrate, EDTA, and serum tubes as appropriate for assessments. The first 2-4 mL of blood will be discarded to avoid spontaneous platelet activation. Blood sampling will be performed at 3 time points as indicated above in the study design section. Various PD assays will be performed as described below:

1. VerifyNow P2Y12
2. Light transmittance aggregometry (LTA)
3. Thrombelastograph Coagulation Analyzer TEG 6s Series system (CORA® system)

Description of laboratory assays

1) VerifyNow (VN) P2Y12: The VerifyNow System is a turbidimetric based optical detection system which measures platelet induced aggregation as an increase in light transmittance (Accumetrics, San Diego, CA) and will be utilized according to manufacturer's instructions, as previously described [14]. The assay is based on microbead agglutination and uses specific reagents for the pathways of interest. The VN-P2Y12 assay, by combining ADP+PGE1, measures changes in

platelet function specific to P2Y12 receptor inhibitors. The assay is based upon the ability of activated platelets to bind fibrinogen. Fibrinogen-coated microparticles aggregate in proportion to the number of GP IIb/IIIa receptors expressed. Microbead aggregation is more rapid and reproducible if platelets are activated; therefore the reagents are incorporated into the assay channel to induce platelet activation without fibrin formation. Light transmittance increases as activated platelets bind and aggregate fibrinogen-coated beads. The instrument measures this change in optical signal and reports results in P2Y12 Reaction Units (PRU). HPR will be defined as PRU>208 and NPR as PRU 85-208 [4].

2) Light transmittance aggregometry (LTA): Platelet aggregation will be performed using LTA according to standard protocols. Blood will be collected in citrated (3.2%) tubes. LTA will be assessed using platelet rich plasma (PRP) by the turbidimetric method in a 2-channel aggregometer (Chrono-Log 490 Model, Chrono-Log Corp., Havertown) as previously described [14]. Platelet agonists will include arachidonic acid (AA, 1 mM), collagen (3µg/ml), ADP (5 and 20 µM), TRAP (15 µM). PRP will be obtained as a supernatant after centrifugation of citrated blood at 1000 rpm for 10 minutes. The isolated PRP will be kept at 37° C before use. Platelet poor plasma (PPP) will be obtained by a second centrifugation of the blood fraction at 2800 rpm for 10 minutes. Light transmission will be adjusted to 0% with the PRP and to 100% for the PPP for each measurement. Curves will be recorded for 6 minutes and platelet aggregation will be determined as the maximal percent change (MPA) in light transmittance from baseline using PPP as a reference. HPR will be defined as MPA with 20 µM ADP > 59% and MPA with 5 µM ADP > 46% [4].

3) TEG 6s Series system (CORA® system): the TEG 6s system (Haemonetics Corporation, Braintree, MA, USA) will be used according to manufacture instructions [15]. In brief, the CORA® system is a new generation portable thrombelastography technology able to evaluate all phases of

hemostasis, including time to clot formation, rate of clot formation, strength of clot and residual clot strength due to antiplatelet drugs, rate of clot lysis. Disposable assay cartridges contain all of the components necessary to allow the analyzer to prepare samples and perform hemostasis tests. The analyzer automatically draws the blood into the active area of the cartridge, meters the exact amount required for the test, and mixes it with the reagents spotted in the cartridge. The analyzer then monitors the harmonic motion of a pendant drop of blood in response to external vibration. As the sample transitions from a liquid state to a gel-like state during clotting, the modulus of elasticity and resonant frequency increase. The instrument measures these variations in resonant frequency during clotting and lysis. The results are displayed in a table and on a graphical tracing that reflects a hemostasis profile of clot formation. The resulting hemostasis profile is a measure of the time it takes for the first measurable clot to be formed, the kinetics of clot formation, the strength of the clot, and the breakdown of the clot, or fibrinolysis. In particular, the PlateletMapping Cartridge are used to assess platelet function in patients who have received platelet inhibiting drugs. The PlateletMapping assay consists of a set of agonists, ADP and AA platelet agonists together with ActivatorF, which can measure the inhibition of platelet function. This assay specifically determines the MA (Maximum Amplitude, a measure of clot strength) and the reduction in MA due to antiplatelet therapy and reports it as a percentage of reduction in clot strength. The assay uses AA and ADP agonists to generate test results that reflect the inhibiting effects of antiplatelet agents such as TxA2 Inhibitors (e.g., aspirin) and ADP $P2Y_{12}$ inhibitors (e.g., clopidogrel). Since thrombin (present in blood samples) is the primary and most potent activator of platelets, its activity must be inhibited with heparin so that the platelet activating effects of ADP and AA can be measured. Since thrombin has been rendered inactive by heparin, activatorF is used to replace thrombin's role in the conversion of fibrinogen to fibrin and Factor XIII to Factor XIIIa. Thus, with this cross-linked fibrin

network as the foundation, additional clot strength due to platelet-fibrin bonding related to ADP and AA platelet receptor activation can be measured. The HKH reagent, a combination of kaolin and heparinase, generates test data for the uninhibited MA resulting from thrombin activation of the blood sample, while the heparinase neutralizes the effects of heparin. The HKH test provides measures of R (Reaction time; the amount of time between the start of the test and the beginning of coagulation), K (the speed of formation of the clot from R time to a specific clot strength), Angle (the speed of clot strengthening), LY30 (Percent lysis 30 minutes after MA is finalized) and MA parameters; The activatorF test provides the contribution of fibrin to the overall strength of the clot. This test value is used in the calculation of aggregation/inhibition for MA ADP and MA AA. The AA and ADP test provide measures of MA, percent inhibition and percent aggregation. HPR will be defined as MA ADP > 47 mm [15].

Genetic testing

Spartan RX rapid genotyping: Spartan RX (Spartan Bioscience Inc., Ontario, Canada) is the rapid genotyping system determining the CYP2C19 (*1,*2,*3,*17) allele status within 1 hour [16]. This test consists of four separate steps intended to be done in less than 8 minutes: acquisition of a buccal swab; insertion of the swab into the cartridge; insertion of the reaction solution into the device; and analysis of CYP2C19 genotype triggered by a button on the device.

Lipid profile and PCSK9 levels

Blood for the evaluation of the effects on lipids will be drawn at the same time points as described above for PD measurements. Cholesterol measures will include a standard lipid profile and lipoprotein(a) [Lp(a)], which will be measured by the central laboratory of our institution

according to standard protocols. At each time point, blood samples samples will also be stored for measurement of PCSK9 serum levels and for possible future analyses. PCSK9 levels will be measured by ELISA as previously described [17].

Study endpoints and sample size calculation

The primary end point of our study is the reduction in platelet reactivity, as defined by PRU, between evolocumab and placebo at 30 days after randomization in each cohort. We hypothesize that evolocumab therapy will be associated with a reduction of 30 PRU in patients with HPR and NPR. The rationale for considering a reduction of 30 PRU is because this value approximates that observed with more intense lipid lower therapy using statin therapy [18]. Statistical assumptions were derived for each cohort of patients based on distribution of their PRU levels from a cohort of subjects on maintenance clopidogrel therapy (HPR: 247 ± 29; NPR: 150 ± 35). In patients with HPR, 17 patients per group (34 total) are needed to detect a 30 PRU difference with 80% power. In NPR patients, 23 patients per group (46 total) are needed to detect a 30 PRU difference with 80% power. Therefore, under the assumption of a 30 PRU reduction with evolocumab compared with placebo in each cohort, 80% power and α of 0.05, 80 total patients with a valid primary end point will be needed. Considering a possible dropout rate of 10%, up to 90 patients will need to be randomized. We anticipate that up to 500 patients will need to be screened to reach our sample size. Given the absence of preliminary studies exploring the effects of PCSK9 inhibitors on platelet aggregation the expected reduction in PRU was arbitrarily defined.

Other endpoints will include the comparisons between evolocumab and placebo of all PD parameters measured by multiple assays, and of cholesterol levels at each time point. Exploratory analyses will assess the differential response to evolocumab treatment according to the presence of

LOF genetic polymorphisms as well as the levels of PCSK9. We will also investigate different response profiles in patients with and without aspirin.

Statistical analysis plan

Categorical variables will be expressed as frequencies and percentages. Continuous variables will be presented as mean ± SD or median [IQR]. An analysis of covariance (ANCOVA) method with a general linear model will be used to evaluate the primary end point as well as all between-groups comparisons of platelet reactivity, cholesterol levels and HS-CRP. The Fisher's exact test or Pearson's chi square test will be used to evaluate t comparisons of HPR and NPR rates between groups.

A 2-tailed p value of < 0.05 will be considered to indicate a statistically significant difference for all the above analyses. Statistical analysis will be performed by our group using SPSS v22.0 software (SPSS Inc. Chicago, IL).

Publication Strategy/Additional Information

Study subjects will be identified first (months 1-24): we expect to enroll 3-4 subjects monthly and complete enrollment in 24 months (total: 90 subjects randomized). Month 25 will be implied for statistical analysis and months 26-27 for manuscript preparation. We intend to present data at a major scientific meeting at completion of the study.

Possible Discomforts and Risk

In clinical trials, the most commonly reported adverse events with evolocumab included nasopharyngitis (4.0% of evolocumab recipients vs. 3.9% of controls), arthralgia (1.8% vs. 1.6%), myalgia (2.0% vs. 1.8%), upper respiratory tract infection (2.1% vs. 2.2%), back pain (2.3% vs. 2.2%), and nausea (1.8% vs. 1.2 %). Injection-site reactions were reported in 3.3% of evolocumab recipients and 3.0% of controls. Allergic reactions occurred in 5.1% and 4.7% of evolocumab-treated and placebo-treated patients, respectively. The most common allergic reactions were rash (1.0% vs. 0.5% for evolocumab and placebo, respectively), eczema (0.4% vs. 0.2%), erythema (0.4% vs. 0.2%), and urticaria (0.4% vs. 0.1%) [19].

All clinical events described above, if they were to occur, as well as death, myocardial infarction, stroke, and urgent revascularization procedure with PCI or coronary artery bypass grafting will be recorded. Clinical events will be evaluated by a local committee, comprised of 2 faculty members (2 cardiologists), not directly involved in the research. In the event of a report of a serious adverse event, the local committee will meet and treatment management will be managed according to physician recommendation.

Definition of Adverse Events

An adverse event is any unintended or undesirable experience that occurs during the course of the clinical investigation whether or not it is considered to be therapy related. This includes any newly occurring event or previous condition that has increased in severity or frequency since the initiation of study treatment. Adverse events will be followed until resolution while the patient remains on-study. Once the patient is removed from study, events thought to be related to the study therapy will be followed until resolution or until the patient starts a new treatment regimen.

Serious Adverse Events (SAE): An adverse event occurring while on study that results in any of the following outcomes:

- Death
- A life-threatening adverse experience.
- A persistent or significant disability, incapacity, or is a congenital anomaly, or birth defect.
- Requires inpatient hospitalization, or prolongation of existing hospitalization.

All SAEs will be collected, recorded and reported, whether or not considered causally related to the study drug, or to the study procedure(s). Study related SAE is defined as SAE occurring while on study and considered related (reasonable possibility that the study treatment caused the adverse experience) to the study treatment.

The definition of serious adverse event also includes 'important medical event'. Medical and scientific judgment should be exercised in deciding whether expedited reporting is appropriate in other situations, such as important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient and/or may require medical or surgical intervention to prevent one of the other outcomes listed in the definition above. These should also usually be considered serious. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse.

All suspected unexpected serious adverse reactions (SUSARs) related or possibly related to evolocumab and their follow-up reports will be reported to Amgen within 24 hours of submission to the regulatory agency, IRB or IEC. A copy of any safety report involving an Amgen drug (e.g. evolocumab) submitted to the regulatory agency, IRB or IEC, will be faxed to Amgen, within 24

hours of such submission. All pregnancies and pregnancies occurring in the partner of a patient participating in the study will be reported within 10 calendar days of Investigator's awareness.

Possible benefits

The present investigation is aimed to evaluate the PD effects of evolocumab as an add-on therapy to standard antiplatelet treatment in patients with CAD. This study is not designed to evaluate differences in clinical benefit. However, differences in antiplatelet profiles may potentially prompt further investigations of the clinical implication of this difference by means of a larger scale clinical study.

Potential Financial Risks or Benefits

None

Conflict of Interest

None

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