Protocol

1. Project Title: Impact of chronic kidney disease on pharmacodynamic profiles of the $P2Y_{12}$ receptor inhibitor clopidogrel in the setting of type 2 diabetes mellitus and coronary artery disease

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3. Abstract: Patients with diabetes mellitus (DM) and coexisting chronic kidney disease (CKD) are at increased risk of atherothrombotic events, underscoring the importance of secondary prevention antiplatelet therapy in these high-risk patients. Clopidogrel is the most widely used platelet $P2Y_{12}$ receptor inhibitor in patients with coronary artery disease (CAD). However, despite its clinical benefits, many patients still experience recurrent atherothrombotic events. This is in part due to the impaired effects of clopidogrel in DM patients, particularly among those with coexisting CKD. However, underlying mechanism(s) leading to magnification of impaired clopidogrel response among DM patients with CKD remain unexplored. The ever growing prevalence of CKD in patients with DM and their high risk of recurrent events underscores the need to define such mechanism(s) as this may set the basis for identifying treatment regimens leading to more effective platelet inhibition and cardiovascular protection in these high-risk patients. The proposed study will test the central hypothesis that in DM patients the presence of CKD reduces clopidogrel-mediated $P2Y_{12}$ inhibitory effects through synergistic mechanisms, which include upregulation of the $P2Y_{12}$ signaling pathway and impaired clopidogrel metabolism. Comprehensive pharmacokinetic and pharmacodynamic assessments, including *ex vivo* and *in vitro* experiments, evaluating the impact of CKD on antiplatelet drug response in DM patients are proposed. This investigation is part of our long-term goal of defining optimized antiplatelet treatment regimens in high-risk CAD patients. The REDCap system will be used to store study related data, and all patients will be identified using a subject identifier.

4. Background: Patients with DM are at increased risk of atherothrombotic events [1,2]. Importantly, DM is a key risk factor for the development of CKD, which further enhances atherothrombotic risk [3-5]. These observations underscore the importance of antiplatelet therapy for secondary prevention of atherothrombotic recurrences in these high-risk patients. Clopidogrel is the most widely used platelet $P2Y_{12}$ receptor inhibitor which, in adjunct to aspirin, reduces ischemic event rates in high-risk patients with CAD [6,7]. However, despite the clinical benefit of adjunctive clopidogrel therapy, DM patients frequently experience recurrent atherothrombotic events [2,8]. This may be in part due to the impaired PK and PD effects of clopidogrel in DM patients which leads to increased platelet reactivity compared with non-DM patients [8-10]. Moreover, studies have also shown patients with CKD to have reduced clinical benefit with clopidogrel [11-14]. However, PD studies provide mixed results on the independent role of CKD as a determinant of impaired clopidogrel effects [15-19]. This may be attributed to confounders intrinsic to the heterogeneous study populations in which these studies have been

performed. In fact, assessments conducted selectively in type 2 DM patients have consistently shown that the presence of impaired renal function markedly reduces clopidogrel-induced antiplatelet effects [15,17]. These PD findings may explain why DM patients with CKD are particularly at increased risk of atherothrombotic recurrences [20]. However, the underlying mechanism(s) leading to magnification of impaired clopidogrel response among DM patients with CKD remains unexplored. The increased cardiovascular (CV) risk of DM patients is in part attributed to increased platelet reactivity and inadequate response to oral antiplatelet agents, including clopidogrel [1,2,6]. Reduced clopidogrel-induced antiplatelet effects among DM patients may in part account for their poor outcomes after percutaneous coronary intervention (PCI) [1,2,8,9,17,21-23]. This is mainly due to an attenuation of clopidogrel's PK profile, characterized by lower active metabolite levels [9,24]. Notably, patients with high platelet reactivity (HPR) while on clopidogrel therapy are at increased risk of ischemic recurrences [25-26]. CKD is a pandemic public health problem [27-29] and patients with all stages of CKD are at increased CV risk, including increased mortality [30-37]. In CAD patients, the presence of either DM or CKD increases long-term CV events to a similar extent. However, when DM and CKD coexist, the risk is further amplified [20]. Mechanisms explaining the enhanced CV risk carried by CKD include lower likelihood to receive evidence-based therapies [38], endothelial dysfunction, platelet activation and reduced response to antiplatelet therapies [14,39]. Subgroup analysis of clinical trials showed a reduced benefit of clopidogrel in CKD patients [14]. High levels of circulating procoagulant factors, increased thrombin generation, abnormalities of nitric oxide synthesis, upregulation of the $P2Y_{12}$ signaling pathway and poor bioavailiability of C-AM have been proposed to explain platelet hyperreactivity among CKD patients [14,39]. Platelets from patients with CKD are exposed to higher levels of dinucleoside polyphosphates, which can up-regulate the platelet $P2Y_{12}$ signaling pathway [40-42]. Further, CKD is associated with reduced activity of the hepatic CYP system, a key mediator of clopidogrel metabolism, which can lead to reduced generation of C-AM [43-44]. Indeed, high platelet reactivity in patients with DM and CKD may be due to a synergistic effect of the two clinical disorders, which contribute to the enhanced risk of CV events when they coexist [20].

Understanding the mechanisms leading to the different pharmacological profiles of $P2Y_{12}$ inhibitors in patients with DM and CKD represents a critical knowledge gap, given the prognostic implications associated with profiles of antiplatelet drug response. Defining the underlying mechanism leading to magnification of impaired clopidogrel response among DM patients with CKD is critical to set the basis of improved secondary prevention in these patients at the highest risk of CV events. The prognostic implications associated with inadequate antiplatelet drug response are noteworthy, given that 30-40% of ACS patients have DM associated with CKD [45] and ischemic recurrences, which are higher in the presence of DM and CKD, represent a major health care burden. In the US more than $150 billion is spent on ACS care, and 60% of the costs are a result of ischemic recurrences [46,47]. The results of the proposed investigation will have the potential to help interpret findings from large scale clinical trials as well as help optimize and personalize antiplatelet treatment in this high-risk setting.

We propose unique and wide-ranging PK and PD assessments with both *ex vivo* and *in vitro* experiments using a broad range of assays to define mechanisms of impaired clopidogrel effects in DM patients with CKD. This is noteworthy because clopidogrel is the most utilized $P2Y_{12}$ receptor inhibitor across the globe and this is the first study specifically and prospectively designed to comprehensively address this area of unmet need.

5. Specific Aims: The proposed study will test the <u>*central hypothesis*</u> that in coronary artery disease (CAD) patients with diabetes mellitus (DM), the presence of chronic kidney disease (CKD) reduces clopidogrel-mediated $P2Y_{12}$ inhibitory effects through synergistic mechanisms, which include upregulation of the $P2Y_{12}$ signaling pathway and impaired clopidogrel metabolism. Our approach is <u>*innovative*</u> since to date there has not been any comprehensive pharmacodynamics (PD) and pharmacokinetic (PK) study, involving both *ex vivo* and *in vitro* experiments, evaluating the impact of CKD on antiplatelet drug response in DM patients. The <u>*study proposed is significant*</u> since it advances our understanding on therapeutic approaches aimed to improve outcomes in a high-risk patient population representing a pandemic health care problem such as those with DM and CKD. Two study aims have been elaborated to reach our goals:
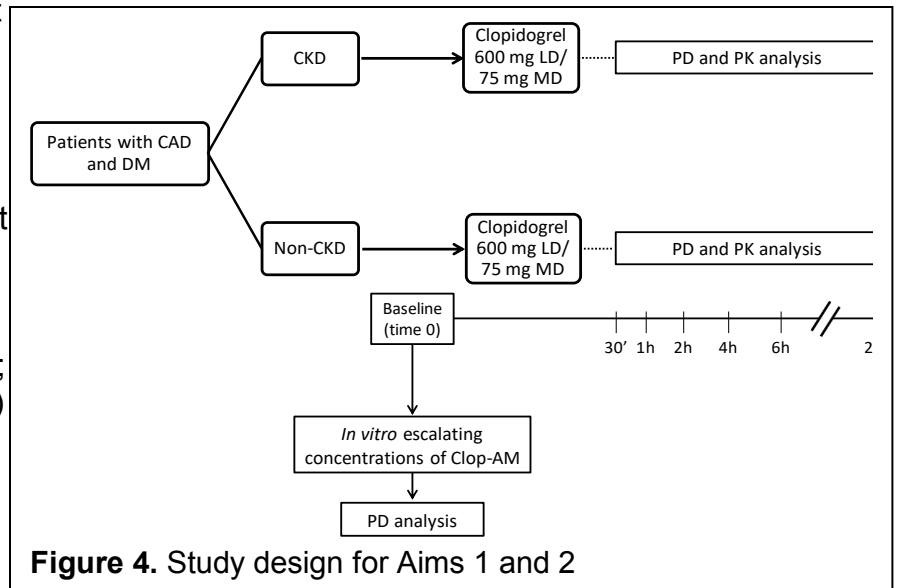
- <u>AIM 1</u>. To assess the functional status of the platelet $P2Y_{12}$ signaling pathway in DM patients with and without CKD. <u>*Study hypothesis*</u>: DM patients with CKD have upregulation of the platelet $P2Y_{12}$ signaling pathway compared to DM without CKD. <u>*Rationale*</u>: Platelets from patients with CKD are exposed to higher levels of dinucleoside polyphosphates, which can act as agonist of $P2Y_{12}$ causing a decrease in VASP (vasodilator-stimulated phosphoprotein) phosphorylation and therefore lead to upregulation of the platelet $P2Y_{12}$ signaling pathway. The <u>*investigational sequence*</u> to pursue this study aim will imply serial and comprehensive PD assessments in DM patients with and without CKD as assessed *ex vivo* following treatment with clopidogrel and *in vitro* following exposure to $P2Y_{12}$ antagonism, in the setting of a prospective study design.

- <u>AIM 2.</u> To assess profiles of clopidogrel metabolism in DM patients with and without CKD. <u>*Study hypothesis*</u>: DM patients with CKD have impaired clopidogrel metabolism. <u>*Rationale*</u>: CKD is associated with reduced activity of the hepatic cytochrome P450 (CYP) system, which is a key mediator of clopidogrel metabolism. The <u>*investigational sequence*</u> to pursue this study aim will imply comprehensive and serial PK assessments of clopidogrel pro-drug and clopidogrel's active metabolite (C-AM; R-130964) after administration of a clopidogrel loading dose (LD) in DM patients with and without CKD.

6. Research Plan: Study Aims 1 and 2 will be pursued through the conduct of a prospective open-label design in which CAD patients with DM on low-dose (81 mg) aspirin will be prospectively recruited. Patients will be stratified according to CKD status into CKD and non-CKD groups. CKD will be defined according to the functional definition of the KDIGO (Kidney Disease: Improving Global Outcomes) guidelines (CKD: glomerular filtrate rate [GFR] <60ml/min/1.73m^2; non-CKD: GFR ≥ 60ml/min/1.73m^2) [53,54]. The rationale for considering the functional classification to initially stratify patients is in line with clinical studies showing the increased CV risk and markedly dysfunctional platelets

according to GFR strata [12,13,15]. For patient stratification, GFR will be estimated through the CKD-EPI (chronic kidney disease epidemiology collaboration) equation, as this is suggested by guidelines as the most accurate method to calculate GFR, especially for values in the normal range (above 60ml/min/1.73m^2) [53,54]. For comparison, kidney function will also be estimated with the MDRD (modification of diet in renal disease) and Cockcroft-Gault equations. In addition, as exploratory analysis, renal function will also be classified according to markers of kidney damage, in particular albuminuria, in order to obtain a more comprehensive information about CKD [53,54]. Albuminuria will be evaluated as albumin-to-creatinine ration (ACR) expressed in mg/g, which is approximately equivalent to albumin excretion rate. According to KDIGO guidelines, CKD will be defined as ACR >30mg/g and non-CKD as ACR ≤30mg/g [53,54]. Albuminuria will be measured on random untimed spot urine samples. Patients' metabolic status and glycemic control will be assessed at baseline by measuring fasting plasma glucose, HbA1c and lipid profile.

After providing informed consent, in the *ex vivo* experimental component of our study, eligible patients will be administered a 600-mg LD of clopidogrel followed by a single 75-mg MD administered after 24 hours; blood samples for PD (Study Aim 1) and PK (Study Aim 2) assessments will be collected at a total of 8 time points: baseline (before LD administration) and 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours and

Figure 4. Study design for Aims 1 and 2

24 hours after LD (before administration of MD), and 2 hours after administration of the 75 mg MD of clopidogrel. In the *in vitro* experimental design of our study (Study Aim 1), blood samples collected at baseline only (before LD administration) will be used; PD testing will be performed before and after incubation (for 30 minutes at 37°C) with escalating concentrations of C-AM (1, 3 and 10 µM). This will allow to specifically explore the functional status of the P2Y$_{12}$ signaling pathway. A flow diagram of the study design is presented in Figure 4. In order to account for possible confounders, CYP2C19 genetic status (the major genetic determinant of clopidogrel metabolism) will be assessed through a rapid assay or by central lab at baseline. Laboratory personnel will be blinded to group assignment.

b) Study population: Patients will be recruited at the cardiology clinics of the University of Florida-Jacksonville. Patients will be approached by the physician and research staff and given information on the study. Consent will be reviewed and the patient will be given time to ask any questions and have all concerns addressed.

Inclusion criteria:

1) Type 2 DM, defined according to ADA definition [55], on treatment with oral hypoglycemic agents and/or insulin 2) age >18 years old; 3) angiographically documented CAD; 4) on treatment with low-dose aspirin (81mg/day) for ≥30 days as part of standard of care.

Exclusion criteria:

1) Use of any antiplatelet therapy (except aspirin) in prior 30 days; 2) use of parenteral or oral anticoagulation; 3) active bleeding; 4) high risk of bleeding; 5) clinical indication to be on a $P2Y_{12}$ receptor inhibitor; 6) end-stage renal disease on hemodialysis; 7) any active malignancy; 8) platelet count < $100x10^6$/μl; 9) hemoglobin <9 g/dl; 10) severe known liver disease; 11) hemodynamic instability; 13) known allergy to clopidogrel; 13) pregnant / lactating females (women of childbearing age must use reliable birth control).

c) Laboratory assessments

Our group has been deeply invested in conducting translational investigations assessing $P2Y_{12}$ receptor inhibitors. This has allowed us to have ample experience with the methodologies described to conduct our study aims, as supported by our description in the Preliminary Studies section and summarized below.

Blood sampling: blood sampling for the *ex vivo* component of our experiment will be performed at baseline and at 7 time points, as described above. For the *in vitro* component of our experiment, blood will be collected at baseline and will be incubated with escalating concentrations (1, 3 and 10 μM) of C-AM (Figure 4).

PD assessments:

(1) VerifyNow PRU: The VerifyNow System is a turbidimetric based optical detection system which measures platelet aggregation as an increase in light transmittance (Accriva, San Diego, CA, USA) [56]. The assay is based on microbead agglutination and uses specific reagents for the pathways of interest. The change in optical signal is reported in PRU. HPR will be defined using consensus definitions, based on cut-off levels associated with an increased risk of recurrent ischemic events, as PRU> 208 [26].

(2) Whole blood vasodilator-stimulated phosphoprotein (VASP): VASP phosphorylation (VASP-P) is a marker of $P2Y_{12}$ receptor activity which will be assessed according to standard protocols [56]. VASP-P levels will be quantified with ELISA method with the Platelet VASP-FCM kit (Biocytex Inc., Marseille, France) and the platelet reactivity index (PRI) calculated. HPR will be defined using consensus definitions as PRI> 50% [26].

(3) Light transmittance aggregometry (LTA): LTA will be assessed using platelet rich plasma (PRP) by the turbidimetric method in a 2-channel aggregometer (Chrono-Log Corp., Havertown, PA, USA) as previously described [56]. Platelet agonists will include 5 and 20 μmol/L ADP. HPR will be defined using consensus definitions as maximal aggregation >59% and >46% with ADP 20 μmol/L and 5 μmol/L, respectively) [26].

PK assessment: determination of C-AM and clopidogrel pro-drug concentrations in plasma will be performed using liquid chromatography with tandem mass spectrometry according to standard protocols as previously described [9]. The geometric mean area under the concentration-time curve through the sampling time of the last quantifiable C-AM concentration ($AUC[0-t_{last}]$), the maximum observed plasma concentration (C_{max}) of C-AM, and the time to reach maximum concentration (T_{max}) will be calculated.

Genetic testing:

Spartan RX rapid genotyping: Spartan RX (Spartan Bioscience Inc., Ontario, Canada) is a rapid genotyping platform able to determine the CYP2C19 (*1,*2,*3,*17) allele status within 1 hour. This test consists of four separate steps: 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 [57-58].

Central lab genotyping: genetic testing to determine the CYP2C19 (*1, *2, *3, *17) allele status will be performed at baseline by central pathology lab by processing a whole blood sample using standard techniques if rapid genotyping is not available.

Dinucleoside polyphosphates levels: Diadenosine polyphosphate (Ap(n)A) content in the supernatant and in intact platelets will be determined at baseline using a chromatographic assay established on the basis of affinity- and reversed-phase chromatographic methods, as previously described [42].

Assessment of metabolic control: Fasting plasma glucose, HbA1c and lipid panel (total cholesterol, HDL, LDL and triglycerides) will be measured at baseline by our central laboratory.

7. Possible Discomforts and Risks: The most common clinical side effects of Clopidogrel are pruritis (itching), purpura (purple colored spots), diarrhea, rash, and bleeding that can be minor, life-threatening or fatal. The risks of the blood draw may include faintness, inflammation of the vein, pain, bruising or bleeding at the site of the puncture.There is also a slight risk of infection from the blood draw.

8. Possible Benefits: There is no guarantee that you will personally benefit by participating in this research study. We hope to improve our knowledge about the effects of Clopidogrel in diabetic patients with and without kidney disease.

9. Conflict of Interest: None