The Effects of Evolocumab on Endothelial and Inflammatory Biocellular Markers in Patients With Diabetes and Atherosclerotic Vascular Disease (METCHNIKOFF)
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THE EFFECTS OF EVOLOCUMAB ON ENDOTHELIAL AND INFLAMMATORY BIOCELLULAR MARKERS IN PATIENTS WITH DIABETES AND ATHEROSCLEROTIC VASCULAR DISEASE (METCHNIKOFF)

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1. RATIONALE:

Atherosclerosis is a chronic inflammatory disease of the arterial wall that involves endothelial cell (EC) dysfunction; monocyte recruitment, retention and activation; and vascular smooth muscle cell (SMC) proliferation.[1] ECs can be activated by diverse physiologic stimuli including cytokines, catecholamines, and shear stress.[2, 3] Persistent activation of EC can lead to vascular dysfunction and systemic inflammation, both of which are known to drive vascular events including stroke.[4, 5] In aortic endothelial cells and vascular SMC, NADPH oxidase dependent reactive oxygen species (ROS) increases PCSK9 expression via lectin LDLR-1 (LOX-1).[6] Atherosclerosis is site specific with increased expression of PCSK9 as in low wall shear stress regions of murine aorta.[7]

Although we appreciate that inflammation is a driver of acute coronary syndromes (ACS) and strokes, profiling circulating inflammatory mediators (e.g. CRP, cytokines, lipoproteins) has not greatly added to traditional management strategies. One possible explanation for this gap is that circulating inflammatory markers capture the systemic inflammatory state of the patient and not local inflammation in specific arterial beds. In animal models, the innate immune system responds to local plaque rupture, leading to the recruitment of circulating monocytes. In human observational studies, elevated levels of monocytes correlate with infarct and stroke size.[8] Although it is clear that circulating monocytes are elevated in patients with atherosclerotic diseases, their molecular profiles and how they relate to clinical atherosclerotic cardiovascular disease remain largely unknown. Furthermore, even though it is known that circulating monocytes can change with acute and chronic inflammatory stresses, it remains unclear how PCSK9 inhibition and subacute reductions in lipoproteins impact circulating monocyte subsets and their functional responses. As sentinels of innate immune system, the circulating monocytes may provide a snapshot into local plaque microenvironment providing a functional "biocellular-marker" that integrates both the systemic and local inflammatory state of patients with atherosclerotic disease.[9]

Although experimental models have linked lipoproteins and EC dysfunction with systemic inflammation, relatively little is known about this network in clinical populations and specifically how it changes with PCSK9 inhibition. Because of PCSK9 effects on the LDL receptor, PCSK9 inhibition is likely to inhibit pro-inflammatory changes in circulating monocyte subsets and functions. In circulating monocytes, toll-like receptors (TLR) sense inflammatory molecules. TLR responses are mediated through MyD88 and TRIF, which leads to activation of signaling pathways including ***Jak-STAT, Akt, AMPK, mTOR, PKA, NFkB,*** and ***MAPK***, which mediate inflammatory transcriptional programs. The participation of PCSK9 in vascular inflammation is supported by preclinical studies using PCSK9 siRNA in macrophages. These studies have demonstrated that PCSK9 inhibition in macrophages reduces NF-kB inflammatory responses.[10]

Furthermore, oscillatory shear stress increases the activation of pro-inflammatory signaling pathways and reduces activation of anti-inflammatory pathways. As an exploratory aim, we will measure low shear blood viscosity to examine the associations between baseline and on-trial change in low shear blood viscosity with inflammatory mediators. The data on blood viscosity will also be used to support our larger aim concerning the putative role of PCSK9 inhibition on cardiac microvascular dysfunction, as blood flow in the microcirculation is highly dependent on changes in blood viscosity.

Our aim is to define how PCSK9 inhibition alters the circulating monocyte populations and to systematically profile the transcriptional and proteomic responses to TLRS and how these responses are modulated by PCSK9 inhibition in the peripheral blood mononuclear cells (PBMC) clinical samples and relate these molecular signatures to clinical and imaging variables**.** The <u>overall rationale</u> for the work proposed is to understand the molecular mechanisms through which PCSK9 and circulating lipoproteins orchestrate TLR response in circulating monocytes. The <u>outcome</u> measures for this aim include PBMC subset analysis and transcriptional and proteomic analysis of inflammatory signaling pathways in

response to TLR stimulation. After the proposed studies for this aim have been completed, *it is our expectation that we will have defined the molecular pathways through which PCSK9 modulates inflammatory responses in circulating monocytes and how these molecular signatures relate to clinical variables known to be important in CVD risk stratification.*

Microfluidic technology has recently been applied to molecular biology and to clinical diagnostics. Because of its small sample requirements and exquisite spatial control, microfluidics is an exciting complement to existing diagnostic technologies. We recently engineered microfluidic-proteomic device that allows for the rapid profiling of immune cell activation (Figure 1). Studies have suggested that understanding the molecular signatures associated immune cell stress responses can be more informative than static measures of inflammation. Profiling the activation patterns of circulating monocytes from clinical patients has been difficult because of the amount and processing of clinical samples required for traditional molecular assays. This microfluidic profiling device will overcome many of these obstacles, allowing us to profile the molecular activation patterns of circulating blood monocytes in parallel with markers of systemic inflammation, lipoproteins, and other clinical variables with the requisite throughput and fidelity. This technology will allow us to link the biological effects of PCSK9 inhibition with immune cell function, providing mechanistic insight into the relationship between lipoprotein metabolism, microvascular dysfunction, and inflammation, ultimately resulting in novel surrogate markers that could result in identification of "at-risk" populations that would benefit from more aggressive LDL cholesterol reduction.

Monocytes are key innate immune system mediators of inflammatory responses and have been implicated as drivers of CVD. Monocyte subsets can be characterized using flow cytometry for surface expression of FcγIII receptor CD16 and the lipopolysaccharide receptor CD14. We will profile monocytes in patients using flow cytometry. Briefly, blood will be collected in Cell Preparation Tubes (CPT) with sodium citrate (BD, New Jersey). Cells will be resuspended in FACS buffer and stained with fluorescently labeled antibodies. Monocyte subsets will be defined using CD14 and CD16. The Nomenclature Committee of the International Union of Immunological Societies defines three monocyte subsets by this method: CD14++CD16- (classical), CD14++CD16+ (intermediate), and CD14+CD16++ (non-classical). Flow cytometric analysis will be performed on a BD Accuri C6 flow cytometer and the data analyzed using FlowJo software (Tree Star, Inc). Monocyte subsets will be defined using CD14 and CD16. Because cholesterol metabolism has been associated with monocyte egress from the bone marrow, we anticipate that PCSK9 inhibition will alter monocyte subsets compared with patients not on therapy.

We will systematically define functional relationships between clinical data, cell-surface markers, and molecular activation of signaling pathways using our microfluidic proteomic platform and PCR arrays. Briefly, circulating monocytes will be isolated and processed as described above. To understand how PCSK9 inhibition modulates transcriptional and signaling responses to inflammatory stimuli, monocytes will be stimulated with TLR ligands and RNA and protein will be isolated at 2 time points (30 min and 2 hours). Inflammatory transcriptional programs will be profiled using PCR arrays (SA Biosciences, Maryland). Activation of inflammatory signaling pathways (MAPK, NFkB, Akt, AMPK, mTOR, Jak-STAT, PKA) will be profiled using our novel microfluidic immunoblotting platform. We anticipate that PCSK9 inhibition will significantly alter TLR activation of signaling pathways and inflammatory transcriptional programs in monocytes. The dynamic responses characteristic of circulating monocytes cannot be captured with typical profiling experiments; we anticipate that by using circulating monocytes as a "biocellular" marker of inflammatory risk, tremendous insight will be gained into how PCSK9 modulates inflammatory responses and these dynamic molecular signatures have the potential to identify patients most at risk of CVD disease and those who would benefit from more aggressive LDL cholesterol reduction.

Circulating biomarkers that interface with inflammation and endothelial function will be measured. These include soluble VCAM-1, ICAM-1, LOX-1, MCP-1 and MIP-1α. Due to the critical role of low wall shear stress

in endothelial dysfunction, inflammation and site-specificity of atherosclerosis, we will measure blood viscosity profiles. Lipoprotein subclasses will be measured by NMR spectroscopy (LabCorp). The choice of NMR is based on a recent study that showed an inverse association between large HDL particle concentration and myocardial perfusion reserve index.[11]

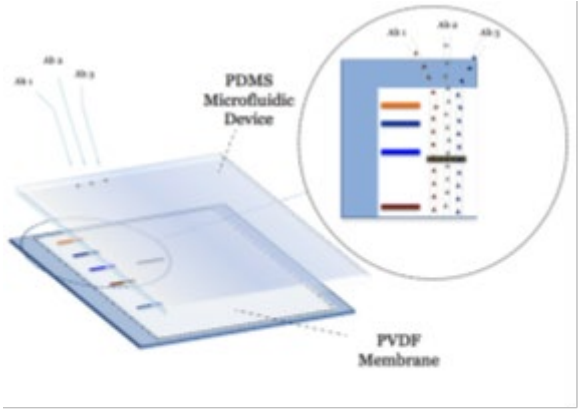

Figure 1| Schematic of the PDMS microfluidic device. This microfluidic device allows high-throughput targeted-proteomic assessment of signaling pathways in individual patient samples.

1.1. Aims:

We therefore propose to evaluate the effects of evolocumab upon biocellular markers potentially altered by PCSK9 inhibition in a population of type 2 diabetes patients with cardiac microvascular dysfunction.

1.1.1. Primary Objective

Determine the ACUTE and SHORT-TERM effects of PCSK9 inhibition with evolocumab on biocellular markers of inflammation, immune mediated thrombosis and rheology. The data from this trial will be used to support a clinical trial to assess the role of PCSK9 inhibition in type 2 diabetes patients with microvascular dysfunction.

1.1.2. Secondary Objective:

a) To define the association between PCSK 9 concentrations and immune-related phenotype.

b) To define the association between Lp(a) concentrations, oxidized phospholipids (OxPL), ApoB, biocellular markers of inflammation, tissue factor and immunothrombosis.

2. SUBJECT ELIGIBILITY

This study can only fulfill its objectives if appropriate subjects are enrolled. In addition to the eligibility criteria listed below, all relevant medical and non-medical considerations will be taken into account when deciding whether an individual subject will be suitable to enter this particular study.

2.1. Inclusion Criteria

a) Subjects ≥ 18 years of age at signing of informed consent

b) A history of clinical ASCVD, which is defined as: acute coronary syndrome, or a history of MI, stable or unstable angina, coronary or other arterial revascularization, stroke, transient ischemic attack (TIA), or peripheral arterial disease presumed to be of atherosclerotic origin.[12]

c) Clinical diagnosis of type 2 diabetes according to ADA/CDA guidelines

d) Subject on stable dose of maximally-tolerated statin therapy for ≥4 weeks prior to screening and LDL-C ≥ 70mg/dL. For subjects whose maximally tolerated dose of statin is no type or dose (i.e. determined to be statin intolerant by primary investigator), background lipid-lowering therapy is not required.

e) Fasting triglycerides ≤ 400 mg/dL (4.52 mmol/L) be central laboratory at screening

f) Willing and able to comply with scheduled visits, treatment plan, laboratory tests and other trial procedures

g) Abnormal urinary Albumin Creatinine Ratio (ACR) as defined by an ACR >2.

2.2. Exclusion Criteria

a) Personal or family history of hereditary muscular disorders

b) NYHA III or IV heart failure, or last know left ventricular ejection fraction (LVEF) < 30%

c) Uncontrolled serious cardiac arrhythmia defined as recurrent and highly symptomatic ventricular tachycardia, atrial fibrillation with rapid ventricular response, or supraventricular tachycardia that are not controlled by medications, in the past 6 weeks prior to randomization

d) Myocardial infarction, unstable angina, percutaneous coronary intervention (PCI) within 6 weeks, coronary artery graft (CABG) or stroke within 3 months prior to randomization

e) Planned cardiac surgery or revascularization

f) Moderate to severe renal dysfunction, defined as an estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73m^2 at screening.

g) Type 1 diabetes, poorly controlled type 2 diabetes (HbA1c > 10%), newly diagnosed type 2 diabetes (within 6 months of randomization), or laboratory evidence of diabetes during screening (fasting serum glucose ≥ 126 mg/dL [7.0 mmol/L] or HbA1c ≥ 6.5%) without prior diagnoses of diabetes

h) Uncontrolled hypertension defined as sitting systolic blood pressure (SBP) > 160 mmHg or diastolic BP (DBP) > 100 mmHg

i) Subject who has taken a cholesterol ester transfer protein (CETP) inhibitor in the last 12 months prior to LDL-C screening, such as: anacetrapib, dalcetrapib or evacetrapib

j) Treatment in the last 3 months prior to LDL-C screening with any of the following drugs: immunosuppresives like cyclosporine, systemic steroids (e.g., IV, intramuscular [IM], or PO) (Note: hormone replacement therapy is permitted), vitamin A derivatives and retinol derivatives for the treatment of dermatologic conditions (e.g., Accutane); (Note: vitamin A in a multivitamin preparation is permitted). Topical retinol prescription and non-prescription derivatives or creams are permitted.

k) Uncontrolled hypothyroidism or hyperthyroidism as defined by thyroid stimulating hormone (TSH) < 1.0 time the lower limit of normal or >1.5 times the ULN, respectively, at screening. Potential subjects with TSH < 1.0 time the lower limit of normal due to thyroid replacement therapy is not considered an exclusion

l) Active liver disease or hepatic dysfunction, defined as aspartate aminotransferase (AST) or alanine aminotransferase (ALT) > 3 times the ULN as determined by central laboratory analysis at screening

m) Known active infection or major hematologic, renal, metabolic, gastrointestinal or endocrine dysfunction in the judgment of the investigator

n) Diagnosis of deep vein thrombosis or pulmonary embolism or major surgical procedures within 3 months prior to randomization

o) Unreliability as a study participant based on the investigator's (or designee's) knowledge of the subject (e.g. alcohol or other drug abuse)

p) Currently enrolled in another investigational device or drug study, or less than 30 days since ending another investigational device or drug study(s), or receiving other investigational agent(s)

q) Female subject who has either (1) not used at least 1 highly effective method of contraception for at least 1 month prior to screening or (2) is not willing to use such a method during treatment and for an additional 15 weeks after the end of treatment, unless the subject is sterilized or postmenopausal;

   a. Menopause is defined as: 12 months of spontaneous and continuous amenorrhea in a female ≥ 55 years old or 12 months of spontaneous and continuous amenorrhea with a follicle-stimulating hormone (FSH) level > 40 IU/L (or according to the definition of "postmenopausal range" for the laboratory involved) in a female < 55 years old unless the subject has undergone bilateral oophorectomy

   b. Highly effective methods of birth control include: not having intercourse or using birth control methods that work at least 99% of the time when used correctly and include: birth control pills, shots, implants, or patches, intrauterine devices (IUDs), tubal ligation/occlusion, sexual activity with a male partner who has had a vasectomy, condom or occlusive cap (diaphragm or cervical/vault caps) used with spermicide

r) Subject who is pregnant or breast feeding, or planning to become pregnant during treatment and/or within 15 weeks after the end of treatment

s) Use of a PCSK9 inhibitor within 10 weeks from screening visit

t) Subject who has any kind of disorder that, in the opinion of the investigator, may compromise the ability of the subject to give written informed consent and/or to comply with all required study procedures

u) Malignancy except non-melanoma skin cancers, cervical or breast ductal carcinoma in situ within the last 5 years

v) Subject who has known sensitivity to any of the products or components to be administered during dosing

w) Subject who is likely to not be available to complete all protocol-required study visits or procedures, and/or to comply with all required study procedures to the best of the subject and investigator's knowledge

x) History or evidence of any other clinically significant disorder, condition or disease (with the exception of those outlined above) that, in the opinion of the principal investigator would pose a risk to subject safety or interfere with the study evaluation, procedures or completion

y) Blood donation 4 weeks prior to screening, or stated intention to donate blood or blood products during the period of the study or within one month following completion of the study

z) Subjects who have participated in other studies within 30 days prior to screening, or have five times the plasma half-life (if known) of the investigational drug, whichever is longer.

aa) BMI >40 kg/m^2

3. STUDY DESIGN

3.1. Study Overview

This is a double-blind, randomized, placebo-controlled, parallel group Phase IV study with two treatment arms: evolocumab SC 420 mg/dL QM or matching placebo. The population will include men and women of non-child-bearing potential with documented CAD and type 2 diabetes.

Approximately 100 subjects will be screened in order to keep 40 subjects to complete the study. Subjects will be followed for 12 weeks during the treatment phase, maintaining the double-blind throughout. Assessments of ACUTE and SHORT-TERM effects of PCSK9 inhibition with evolocumab on biocellular markers of endothelial function will be measured at baseline, Week 2, and Week 12. Safety assessments will be undertaken at each study visit. Subjects whose maximally tolerated dose of statin is no type or dose (i.e. determined to be statin intolerant by primary investigator) must be randomized fairly between 2 treatment arms to prevent selection bias.

Subjects should continue on their stable dose of anti-hyperglycaemic therapies during the course of the study. However, it is important that the haemoglobin A1c (HbA1c) does not rise above 10% and that the patient does not develop severe hypoglycaemia or frequent episodes of mild-moderate hypoglycaemia, as defined by the Canadian Diabetes Association 2008 Clinical Practice Guidelines for the Prevention and Management of Diabetes.[13]

3.2 Randomization Procedure

A randomization list will be produced using a validated system that automates the random assignment of treatment arms to randomization numbers in the specified ratio. At Visit 2, eligible subjects will be given the lowest available randomization number. This number assigns the patient to one of the treatment arms.

3.3 Study Medication

The blinded study medication will consist of:

Evolocumab 420 mg SC QM

Matching Placebo SC QM

The study will utilize the SureClick® auto-injection formulation of evolocumab (formulated with and without the active ingredient). Three doses (140mg per dose) will be administered at each time point.

3.4 Blinding

The identity of the treatments will be concealed by the use of matching placebo to the study drug that are identical in packaging, labeling, appearance and schedule of administration.

3.5 Premature Interruption or Withdrawal from the Study

3.5.1 Premature Interruption of Study Treatment

Subjects have the right to discontinue study treatment for any reason. However, unless they withdraw consent and are no longer willing to participate, they should be followed by telephone calls for the remainder of the trial and all serious events must be reported, regardless of whether or not the event is of cardiovascular origin or occurs under the care of another physician or institution.

In case of a temporary interruption of study treatment, all efforts should be made to re-institute study treatment as soon as the clinical condition of the subject has stabilized based on the judgment of the Investigator.

3.5.2 Withdrawal of Consent

Subjects may decide to fully or partially withdraw their consent to participate in the study. At that point, it should be established, whether the withdrawal is related to study treatment, further assessments, or any further involvement in the study. If the withdrawal is primarily related to study treatment or specific assessments, subjects should be encouraged to continue follow-up by telephone calls and to attend all other subsequent study assessments. Reports on serious events should be collected until completion of the study for all withdrawals, unless the subject objects to such follow-up.

4 SCHEDULE OF STUDY PROCEDURES

Screening assessments and study procedures outlined in this section and in Table 1 can only be performed after obtaining written informed consent.

All on-study visits and dosing should be scheduled from Day 1 (first IP administration). For example, the Week 2 visit is 2 calendar weeks after the study Day 1 visit, which corresponds to study Week 2. When it is not possible to perform the study visit at the specified time point, the visit should be performed within the visit window specified in Table 1. If a study visit is missed or late, including visits outside the visit window, subsequent visits should resume on the original visit schedule. Missed assessments at prior visits should not be duplicated at subsequent visits. With the exception of screening, all study procedures for a visit should be completed on the same day if possible.

Visit 1: Screening

- Written informed consent
- Demographics
- Medical history
- Vital signs
- 12 lead ECG measument
- Concomitant therapy
- Dietary instruction; medication compliance reminder
- Physical examination
- Body height, waist circumference
- Body weight
- Seattle Angina Questionnaire 7 (SAQ7)
- Laboratory assessments: TSH, hematology (CBC), blood chemistry (creatinine/eGFR and electrolytes), Liver Function Tests (ALT, AST), creatinine/eGFR, $HbA_{1C,}$, local lipid panel, urine albumin to creatinine ratio (ACR), urine pregnancy test (if female of child bearing potential)

Visit 2 (Randomization) 7-28 days after Screening

- Review for AEs/SAEs/CV events
- Concomitant therapy
- Vital signs
- Dietary instruction; medication compliance reminder
- Randomization
- Seattle Angina Questionnaire 7 (SAQ7)
- Laboratory assessments: fibrinogen, whole blood viscosity analysis, NMR lipoprotein subclass analysis, Apo B, lipoprotein (a), biocellular marker analysis, Oxidized lipid Analysis
- DNA/mRAN test for lipoprotein and inflammatory polymorphisms that may influence the response to therapy (the specimens will be stocked at site and the tests will be performed later).
- SC IP administration, QM (in-clinic) and 30 minute post-injections observation

Visit 3 (Week 2) ±4 days

- Review for AEs/SAEs/CV events
- Concomitant therapy
- Vital signs

- Dietary instruction; medication compliance reminder
- Seattle Angina Questionnaire 7 (SAQ7)
- Laboratory assessments: hematology (CBC), blood chemistry (creatinine/eGFR, electrolytes), Liver Function Tests (ALT/AST), fibrinogen, whole blood viscosity analysis, NMR lipoprotein subclass analysis, Apo B, lipoprotein (a), biocellular marker analysis, Oxidized lipid Analysis

Visit 4 (Week 4) ±7 days

- Review for AEs/SAEs/CV events
- Concomitant therapy
- Vital signs
- Dietary instruction; medication compliance reminder
- Seattle Angina Questionnaire 7 (SAQ7)
- SC IP administration, QM (in-clinic) and 30 minute post-injections observation

Visit 5 (Week 8) ±7 days

- Review for AEs/SAEs/CV events
- Concomitant therapy
- Vital signs
- Dietary instruction; medication compliance reminder
- Seattle Angina Questionnaire 7 (SAQ7)
- SC IP administration, QM (in-clinic) and 30 minute post-injections observation

Visit 6 (Week 12) ±7 days

- Body height, waist circumference
- Body weight
- Review for AEs/SAEs/CV events
- Concomitant therapy
- Dietary instruction; medication compliance reminder
- Seattle Angina Questionnaire 7 (SAQ7)
- Laboratory assessments: hematology (CBC), blood chemistry (creatinine/eGFR, electrolytes), Liver Function Tests (ALT/AST), fibrinogen, whole blood viscosity analysis, NMR lipoprotein subclass analysis, Apo B, lipoprotein (a), biocellular marker analysis, Oxidized lipid Analysis

Table 1. Schedule of Assesments

Schedule of Assessments	Screening	Baseline/ Randomization	Week 2	Week 4	Week 8	Week 12
Visit Number	**1**	**2**	**3**	**4**	**5**	**6**
Visit Window		**± 7-28 Days**	**± 4 Days**	**± 7 Days**	**± 7 Days**	**± 7 Days**
Informed consent	X					
Demographics & Medical history	X					
Vital signs (HR, BP)	X	X	X	X	X	X
ECG	X					
Review for AEs/SAEs/CV events		X	X	X	X	X
Concomitant therapy	X	X	X	X	X	X
Dietary instruction; medication compliance reminder	X	X	X	X	X	
Physical examination	X					
Body height, waist circumference	X					X
Body weight	X					X
Seattle Angina Questionnaire 7 (SAQ7)	X	X	X	X	X	X
Placebo Injection	X					
Laboratory Analyses:						
TSH	X					
Hematology (CBC)	X		X			X
Chemistry (creatinine/eGFR, electrolytes)	X		X			X
Liver Function Tests (AST, ALT)	X		X			X
Urine albumin to creatinine ratio (ACR)	X					X
$HbA1_c$	X					
Local Lipid Panel	X					
Urine pregnancy (if applicable)	X					
Fibrinogen		X	X			X
lipoprotein(a), Apo B		X	X			X
Whole Blood Viscosity Analysis		X	X			X
NMR Lipoprotein Subclass Analysis		X	X			X
Biocellular Marker Analysis		X	X			X
Oxidized lipid Analysis		X	X			X
DNA/mRNA test		X				
Randomization		X				
Investigational Product: SC IP Administration, QM		X		X	X	

5 CLINICAL PROCEDURES AND SAFETY EVALUATIONS

5.1 Informed Consent

Once deemed appropriate candidates for the study, the subject will be informed of the possibility of study participation. The benefits and risks of participating in the study will be explained to the subject, and the subject will be provided an opportunity to read the informed consent form and ask any questions he/she may have. Prior to conducting any study-related procedures, the subject must provide consent to participate by signing the Institutional Review Board/Research Ethics Board (IRB/REB) approved consent form.

5.2 Demography

This includes age, sex and race.

5.3 Medical History

Data will be collected from subjects at baseline consisting of demographics, history, previous cardiac investigations, previous cardiac history, and current medication.

5.4 Physical Examination

A routine physical examination is required at Visit 1. Any abnormality must be recorded.

5.5 Vital Signs

Vital signs including blood pressure and heart rate will be recorded at the intervals indicated on the schedule of study procedures in Table 1.

5.6 ECG

A 12-lead ECG will be performed at screening visit.

5.7 Body weight, Height, and Waist Circumference

Body weight, height, and waist circumference will be recorded.

5.8 Laboratory Tests

5.8.1 Local Laboratory Tests

Local laboratory assessments include TSH, hematology, blood chemistry, liver function tests (LFTs), eGFR, HbA_{1C}, fibrinogen and urine ACR, Apo B, lipoprotein(a). Please see Table 1 for the exact schedule of all laboratory tests.

5.8.2 Additional Laboratory Tests

The following labs will be performed centrally: whole blood viscosity, lipoprotein subclass, and biocellular marker analyses. In addition, blood samples will be retained and frozen for future analysis including mRNA and DNA analysis.

5.8.3 Biocellular Marker Assessments

We will evaluate the impact on evolocumab on biomarkers of endothelial function. We will assess biomarkers of oxidative stress (MDA), inflammation (MPO), cytokines (IL-6, IL-18 and TNF-α) and vascular endothelial activation (PECAM, ICAM, VCAM and alpha5/beta 3 activations).

5.9 Concomitant Therapy

Medications are not altered for the purpose of this trial, however, any medication changes during the study must be recorded, as must those started before randomization and continued thereafter. During the hospital stay and at following study visits, changes in concomitant therapy must be recorded.

5.10 Study Drug Accounting

Double-blind study medication may only be dispensed and administered according to the study protocol by authorized personnel, as documented in the investigator's trial staff list. At each visit, compliance will be checked and recorded in the case report form.

6 MANAGEMENT OF ADVERSE EVENTS

6.1 Adverse Event (AE) Reporting

An adverse event (AE, AEs) is defined as any untoward medical occurrence in a subject who has been administered a pharmaceutical product and which does not necessarily have to have a causal relationship with this treatment. AEs include those reported spontaneously by the subject and those noted incidentally or as observed by the investigator or study staff.

Study staff will assess all AEs that occur during the period from signing of consent until end of study participation and document these in the source documents. Investigators will evaluate any changes in laboratory values and physical symptoms/signs and will determine if the change is clinically significant. If clinically significant and unexpected adverse experiences occur, they will be recorded in the case report form (CRF).

6.2 Serious Adverse Events (SAEs) and Serious Adverse Drug Reactions (SADRs)

A Serious Adverse Event is defined as any untoward medical occurrence that at any dose results in:

1) Death
2) A life-threatening situation (subject was at risk of death at the time of the event. This does not refer to an event that might have caused death if it was of greater intensity.)
3) New in-patient hospitalization or prolongation of an existing hospitalization
4) Persistent or significant disability or incapacity
5) Congenital anomaly or birth defect
6) Important medical events that may not result in death, be life-threatening, or require hospitalization but may jeopardize the subject and may require medical or surgical intervention to prevent one of the above outcomes (based upon appropriate medical judgment), e.g., allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

Medical and scientific judgment should be exercised in deciding whether other conditions should also be considered serious, such as important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the subject or may require intervention to prevent one of the other outcomes listed above. These should also be considered serious. Follow up information regarding SAEs will be pursued until the event has resolved (with or without sequelae), until death, or until 30 days after the final study visit (whichever comes first.). For any deaths where there is uncertainty about the cause of death, site investigators may request an autopsy if appropriate.

6.3 Reporting Serious Adverse Events (SAE)

Any SAE, including death due to any cause, which occurs between enrolment and the final follow up visit whether or not related to the study drug, must be reported immediately (within 1 business day of the study site's knowledge of the event). The report will contain as much available information concerning the SAE to enable a report to be produced that satisfies regulatory reporting requirements.

The term "severe" is often used to describe the intensity (severity) of a specific event (as in mild, moderate, or severe pain); the event itself, however, may be of relatively minor medical significance (such as severe headache). By contrast, the term "serious" is used to describe an event based on an event outcome or

actions usually associated with events that pose a threat to a subject's life or functioning. Seriousness (not severity) serves as a guide for defining regulatory reporting obligations.

For all collected SAE's, the clinician who examines and evaluates the subject will determine the event's causality based on temporal relationship and his/her clinical judgment. The degree of certainty about causality will be graded using the categories below:

Definitely Related: There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out.

Probably Related: There is evidence to suggest a causal relationship, and the influence of other factors is unlikely.

Possibly Related: There is some evidence to suggest a causal relationship. However, the influence of other factors may have contributed to the event.

Unlikely: A clinical event, including an abnormal laboratory test result, whose temporal relationship to drug administration makes a causal relationship improbable and in which other drugs or chemicals or underlying disease provides plausible explanations

Not Related: The S*AE is completely independent of study drug administration, and/or evidence exists that the event is definitely related to another etiology.*

Pre-existing conditions should be recorded upon patient enrolment (including start date of the condition, and severity - mild, moderate, severe). After the patient signs the informed consent form, any worsening of these conditions would be recorded.

Any new conditions would be recorded including date of onset, date of resolution, severity (mild, moderate, severe, or serious as defined above) and possible relationship to study drug or procedure. As part of the source notes, follow up clinical assessments, laboratory tests, ECGs and diagnostic imaging related to adverse event should be documented.

6.4 Reporting of Serious Unexpected Adverse Drug Reactions (SUADRs)

The PI (or an authorized representative) is responsible for submitting reports of SUADRs to the appropriate regulatory body within the required reporting period. All investigators participating in ongoing clinical studies with the study drug will be notified by the Coordinating Center (or an authorized representative) of all SUADRs that require prompt submission to the REB/IRB. Investigators are responsible for notifying the REB/IRBs in writing of the SUADRs within the required reporting timelines. Copies of the notification will be maintained by the investigator in the study documentation files. Sites will receive detailed reporting guidelines for the SAE reporting process.

7 STATISTICAL ANALYSIS AND SAMPLE SIZE CALCULATION:

7.1 Sample Size Estimation

The goal of this study is to estimate biologically plausible effects as a result of PCSK9 inhibition in a population of patients with type 2 diabetes and microvascular dysfunction. As a result, this will be treated as a "signal" finding trial – and used as preliminary data to support a formal sample size estimation for a larger clinical trial. We anticipate that 40 subjects will be required to provide sufficient data to adequately identify robust changes in the large range of biocellular markers being assessed.

7.2 Statistical Analysis

As we seek to address both early and late changes in biocellular responses as a result of PCSK9i, we will compare changes between week 2 and week 12 with baseline. We will also compare week 2 with week 12 to identify how the expected rheological changes may impact biocellular responses.

7.3 Primary efficacy analysis

The primary efficacy comparison between groups will be by means of linear regression (ANCOVA model). The point estimate of the effect and 95% confidence interval will be obtained, after adjustment for the baseline values.The primary analysis will be ITT. These analyses are viewed as complementary or exploratory, so the interest will be on the estimated treatment effects more than actual hypothesis tests.

Protein immunoblots will be analyzed using ImageJ software. Gene expression will be analyzed using comparative Ct method (ΔΔCt) and normalized to GAPDH.

8 DATA MANAGEMENT

All study data will be stored in an electronic database system, eSOCDAT, which is created and managed by SOCAR Research SA. Study personnel needing access will have their own Login/Password. Access to clinical study information will be based on individuals' roles and responsibilities. The application provides hierarchical user permission for data entry, viewing, and reporting options.

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