PROTOCOLO

TITLE

EFFECT OF PRALUENT® ON LIPOPROTEIN FEATURES CONFERRING CARDIOVASCULAR RISK TO TYPE 2 DIABETIC PATIENTS

ACRONYM

ALLIED (ALirocumab on LIpoproteins to Encounter Diabetes)

TEAM

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BACKGROUND

Approximately 40% of cases of myocardial infarction occur in patients with normal lipid concentrations. Type 2 diabetic patients are a paradigm of this situation since cardiovascular disease is their main cause of death being LDL cholesterol normal or only moderately elevated.

Over the past few years, we and others have demonstrated that cardiovascular risk in these patients is better explained by other characteristics of lipoproteins such as particle abundance or particle diameter. Actually, we have reported how the proprotein convertase subtilisin/kexin type 9 (PCSK9) concentration modulates lipoprotein features towards a more atherogenic profile in type 2 diabetic patients (1). This is remarkable since PCSK9 gene it was first described to regulate cholesterol homoeostasis by accelerating low-density lipoprotein receptor (LDLR) degradation resulting in the decreased catabolism of low-density lipoprotein (LDL) leading to hypercholesterolemia (2).

The PCSK9 inhibitors have changed the paradigm for lipid-lowering therapy to prevent cardiovascular disease. The preliminary results from the FOURIER Study (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk), the first completed outcome study with a PCSK9 inhibitor, showed the clinical benefit of lowering LDL cholesterol below the current goal.

Therefore, since we have reported that PCSK9 modulates lipoprotein profile in diabetic patients (1) and considering that PCSK9 inhibition also modifies lipoprotein kinetics (3, 4), we hypothesize that treatment with Alirocumab may also affect other characteristics of lipoproteins. We propose to study the net electrical charge; the sialic acid proteoforms of apolipoprotein C-III; and the lipopolysaccharide content.

The net electrical charge of the lipoproteins may be considered a new independent contributor to subclinical atherosclerosis, and we have recently demonstrated this in lupus patients (5).

Apolipoprotein C-III is associated with increased atherosclerosis and type 2 diabetes pathogenesis by affecting circulating TG levels and by inducing insulin resistance in pancreas (6). Mature apoC-III undergoes glycosylation in the Golgi apparatus prior to incorporation in lipoproteins. The apolipoprotein C-III (apo C-III) proteoform containing two sialic acids residues (apo C-III2) has different effects on lipid metabolism compared with asialylated (apo C-III0) or the most abundant monosialylated (apo C-III1) proteoforms. There is a strong and inverse relationship between the relative abundance of apo C-III2 proteoform and plasma triglyceride concentrations and proatherogenic lipid profiles in individuals with abnormal glucose metabolism (7).

Lipopolysaccharides (LPS) are the major component of gram-negative bacterial outer membranes. Systemic and chronic exposure to these endotoxins can induce subclinical chronic inflammation. This has been related with an increased metabolic risk. Once in the bloodstream, they bind to lipoproteins, taking part in the humoral detoxification mechanism, as well as in atherosclerosis pathogenesis (8).

HYPOTHESIS

The PCSK9 inhibitor Praluent® has additional benefits on the lipid profile, others than significantly decreasing LDL cholesterol.

OBJECTIVES

Our objective is to study to what extend treatment with Praluent® modifies the following parameters:

- Primary endpoint:

    - Particle number and diameter of 9 lipoprotein subclasses assessed by 2D-NMR.
- Secondary endpoints:
    - VLDL, IDL, LDL and HDL net electrical charge
    - Total, VLDL, IDL, LDL and HDL apolipoprotein C-III content, plus relative amounts of apoC-III sialylated proteoforms (with none, 1 or 2 molecules of sialic acid)
    - Total, VLDL, IDL, LDL and HDL content of lipopolysaccharide.

METHODOLOGY

STUDY PARTICIPANTS

Retrospective, open label study to compare the levels of advanced parameters related with lipoproteins, before and after treatment with Praluent® following the clinical practice. It will be conducted in 100 patients with hypercholesterolemia and type 2 diabetes, who will be recruited in Hospital Sant Joan de Reus and Hospital Virgen del Rocio in Sevilla. We will include those patients, men and women between 18 and 65 years old, prescribed for Praluent®, that agreed a follow-up after 1 month. As exclusion criteria we have considered pregnancy; having known adverse reactions to the treatment; and being under Praluent® therapy before. All participants will sign the informed consent.

Blood samples will be collected before and after 1 month of treatment with Praluent® 150mg subcutaneous every 2 weeks, and plasma samples will be sent to our lab at the Faculty of Medicine, Universitat Rovira i Virgili.

2D-NMR PROFILING

Plasma samples before and after the treatment with Praluent®, will be subjected to the Liposcale test. This advanced lipoprotein test provides with the distribution of 9 lipoprotein plasma subclasses accounting for a more detailed and informative lipid profile which is based on 2D diffusion-ordered 1-H+ nuclear magnetic resonance (NMR) spectroscopy. This method adds diffusion coefficients to classical NMR determinations to provide a direct measure of mean particle size and number for each lipoprotein fraction. This technique also provides the mean particle size and concentration of three sub-fractions of each lipoprotein class (large, medium and small).

ULTRACENTRIFUGATION

Plasma lipoproteins before and after the treatment with Praluent®, will be separated by sequential preparative ultracentrifugation, using a Kontron 45.6 fixed-angle rotor in a

Centrikon 75 (Kontron Instruments, Italy). The lipoprotein fractions isolated will be VLDL (d<1.006 g/mL), IDL (d=1.006–1.019 g/mL), LDL (d=1.019–1.063 g/mL), and HDL (d=1.063–1.200 g/mL).

NET ELECTRICAL CHARGE

The net electrical charge will be determined in circulating lipoproteins (VLDL, IDL, LDL and HDL) before and after the treatment with Praluent®, by a dynamic light scattering technique using a Zetasizer nano ZS instrument (Malvern Instruments, Worcestershire, UK). Each measurement will be averaged over 10 runs of 10 seconds each one. The results will be expressed as the average of each run. Surface lipoprotein charge or zeta potential values will be determined by Laser Doppler Electrophoresis using the same equipment. Samples will be prepared by diluting 1:100 the lipoprotein subfraction in ultrapure water. Measurements will be performed in triplicate with automatic duration runs. Size and zeta measurements will be performed at 25 ºC. The data will be analyzed by zetasizer software (DTS, nano series, version 7.02, Malvern).

APOC-III SIALYLATED PROTEOFORMS

Apo C-II proteofroms will be determined in plasma and as part of the circulating lipoproteins: VLDL, IDL, LDL and HDL, before and after the treatment with Praluent®.
Analysis of apo C-III will be performed using immunoprecipitation of apo C-III complexes coupled to a mass spectrometric analysis on the intact apo CIII protein. In short, “affinity columns” will be prepared by immobilizing the biotinylated antibody (2.5 µg of anti-apoC-III) with streptavidin magnetic beads (Dynabeads, Life Technologies, Grand Island, NY). Following sample preparation, a total of 120 µL of plasma sample or lipoprotein subfractions, diluted 120-fold in PBS, 0.1 % Tween will be incubated for 45 min with the magnetic beads coated with the streptavidin-biotin anti-apo CIII antibody, in order to capture apoC-III proteoforms from each analytical sample. After rinsing the non-specific bounded proteins, captured apolipoproteins will be eluted directly onto a 96-well formatted MALDI target using Sinapinic acid matrix. Linear mass spectra will be acquired from each sample spot using an UltrafleXtrem III MALDI-TOF/TOF instrument (Bruker, Germany) operating in positive ion mode. An average of 3000 laser shots mass spectra will be saved for each sample spot. Mass spectra will be internally calibrated using protein calibration standard-I, and further processed with Flex Analysis 3.0 software (Bruker Daltonics). All peaks representing apoC-III and their proteoforms will be integrated. To reduce the variation due to less precisely captured low-abundant proteoforms, major proteoforms will also be expressed as ratios to the most abundant proteoform. To assess for the consistency of the ionization efficiency and reproducibility

between and within runs, a quality control plasma sample will be run in triplicate with each analysis.

LIPOPOLYSACCHARIDE (LPS)

Lipopolysaccharide will be determined in plasma and as part of the circulating lipoproteins: VLDL, IDL, LDL and HDL, before and after the treatment with Praluent®.

Conjugated fatty acids 3-Hidroxil (3-OHFAs) will be used as biochemical markers of endotoxemia, as structural part of LPS. 3-OHFAs will be determined by GC-QqQ/MS2 mass spectrometry, briefly; an alkaline hydrolysis of LPS followed by a gas chromatography analysis. 3-OHFAs derived from mitochondrial β–oxidation could interfere in the analysis, so, LPS 3-OHFAs will be obtained by the rest between total 3-OHFAs and free 3-OHFAs. Finally, LPS determination will be calculated as the sum of LPS 3-OHFAs determined on each individual, divided by 4; assuming 4 3-OHFAs molecules by each LPS.

STATISTICAL ANALYSIS

The main objective of the study is to compare each of the 23 lipoprotein parameters before and after treatment with Praluent®.

Differences will be assessed with the paired t test or the Mann–Whitney U test depending or whether the variable distribution is normal or not. As we anticipate a certain degree of heterogeneity among participants, adjustment for critical covariates will be applied.

The secondary objective of the study is to assess in the Di@bet.es cohort the association of each of the 23 measured lipoprotein parameters with clinical characteristics of type 2 diabetes. For that purpose, partial correlations adjusted for appropriate covariates will be used. Multivariate logistic regression will be used to investigate a potential role of individual parameters or a combination of them on the prevalence, incidence or evolution of type 2 diabetes.

All the statistical analysis will be conducted on SPSS statistical package (version 25).

BUDGET

Sample transport and Biobank storage n=200 (n=100 before and after the treatment)	2,000
2D-NMR determination n=200 (n=100 before and after the treatment)	10,000
Plasma ultracentrifugation n=200 (n=100 before and after the treatment)	1,500
Zeta determination n=1000 (n=100 before and after the treatment and x5 because it is determined in plasma and in lipoproteins)	5,500
Glycoforms of APOC3 determination n=1000 (n=100 before and after the treatment and x5 because it is determined in plasma and in lipoproteins)	21,000
LPS determination n=1000 (n=100 before and after the treatment and x5 because it is determined in plasma and in lipoproteins)	20,000
TOTAL	60,000 €

WORK PLAN

	2019				**2020**				**2021**			
Participants identification and recruitment	X	X	X	X								
Sample transport				X	X							
2D-NMR determination						X	X					
Plasma ultracentrifugation						X	X					
Net electric charge determination								X				
Sialylated proteoforms of APOC3 determination								X	X			
Lipopolysaccharide determination								X	X			
Analysis										X	X	X

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