

**To:**

Working Group on Coronary Pathophysiology & Microcirculation of the European Society of Cardiology  
The European Heart House  
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**Mobility Travel Grant Report**

Dear Working Group Members,

I would like to thank the Working Group on Coronary Pathophysiology & Microcirculation and the European Society of Cardiology for awarding me the Mobility Travel Grant 2020. It was a great honour to do research at the Cardiovascular Program ICCC of the Research Institute – Hospital Santa Creu I Sant Pau (ICCC-IR-HSCSP) in Barcelona, Spain, and, shortly, be a part of the wonderful research team of Prof. Dr. Lina Badimon, a team doing miracles in the field of cardiovascular diseases research.

In the following report, I will summarize the outcome of my stay at the institute. During my stay at the ICCC-IR-HSCSP, I acquired many new skills and learned how to isolate and purify the different lipoprotein sub-types from plasma, as well as how to perform several lipoprotein functional tests that would help in risk-stratification, diagnosis and evaluation of treatment efficacy when performed in patients with high risk of cardiovascular diseases.

Another gain from this experience has been the possibility to make several acquaintances in the cardiovascular field of research, allowing for possibilities of further collaboration on the topic of question.

Cardiovascular disease (CVD), of which atherosclerotic CVD (ASCVD) is the major component, is responsible for >4 million deaths in Europe each year. Therefore, prevention at population or individual level, aimed to eliminate or minimize the impact of CV diseases and their related disabilities, is a key step towards reducing that prevalence<sup>1</sup>. Current guidelines on CVD prevention recommend total CVD risk assessment in apparently healthy individuals, given the multifactorial nature of ASCVD. Primary, as well as, secondary CVD prevention should be adapted to the total CV risk: the higher the risk, the more intense the measures taken should be<sup>2</sup>.

Statins, the inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, decrease LDL-C, thereby reducing CV morbidity and mortality, as well as the need for coronary artery interventions<sup>3</sup>. Statins, at doses that effectively reduce LDL-C by at least 50%, also seem to halt progression or even contribute to regression of coronary atherosclerosis<sup>4</sup>. Therefore, statins are considered as drugs of first choice in patients with hypercholesterolaemia or combined hyperlipidaemia.

Rosuvastatin is a potent statin with pharmacologic and pharmacokinetic advantages, compared to other statins. Its high affinity for OATP-1B1 ensures a high hepatocyte concentration which results in superior efficacy at lowering LDL-C and TG, as well as improving HDL-C and ApoB:ApoA-1 ratio. Rosuvastatin is a synthetic drug, with relatively low lipophilicity leading to only minimal entry into peripheral cells. This, coupled with its minimal CYP450 metabolism confers relatively better tolerability, safety and drug interaction profile<sup>5</sup>. Several cardiovascular outcome studies have confirmed the beneficial effects of rosuvastatin treatment. The JUPITER study showed

reduction in cardiovascular events and all cause mortality with rosuvastatin, in primary prevention, in patients with lower cardiovascular risk. This is the only statin that has been shown to reduce cardiovascular and all cause mortality<sup>6</sup>.

Although statins are highly effective, even those individuals who have achieved significant LDL-C reductions, with intensive statin therapy, may still experience CV events, referred to as 'residual risk'. This risk is particularly high in certain patients with comorbidities, such as diabetes or atherosclerosis affecting multiple vascular beds. Some of this risk may be addressed by earlier initiation of statin treatment and better blood pressure and diabetes management, or it may require additional lipid-modifying therapies<sup>7</sup>.

Given all the previous, there is a constant need to prove value of new biological assays and biomarkers for better CVD risk-stratification, treatment eligibility prediction and therapy efficacy monitoring.

During my internship at the ICCC-IR-HSCSP, I learned the technique of lipoprotein isolation and purification from plasma using the gradient-density ultracentrifugation method, which separates the lipoproteins based on their density differences. Chylomicrons (density < 1.006 g/mL), VLDLs (density range 1.006-1.019 g/mL), LDLs (density range 1.019–1.063 g/mL) and HDLs (density range 1.063–1.210 g/mL) were obtained from individual plasma-EDTA samples of 40 patients before and after a 3-month rosuvastatin treatment, by sequential ultracentrifugation. Patients were healthy subjects with medium-to-high CV risk, from the University Clinic for Cardiology, Skopje, Republic of North Macedonia. Clinical and biochemical characteristics of all patients before and after rosuvastatin treatment are available and are currently used for the statistical analysis.

In short, lipoprotein fractions were obtained in a Beckman L-60 ultracentrifuge with a fixed-angle type 50.4 Ti rotor (Beckman, Brea, CA, USA). Chylomicrons (the first separated fraction) were obtained at 225,000× *g* for 30 min, whereas each of the other isolated fractions, VLDL/IDL; LDL, HDL, required 18-hour centrifugation periods for their correct purification.

**The first two weeks** of my stay at the ICCC-IR-HSCSP were devoted to the purification of the lipoprotein fractions in all samples (40 patients before/after statin treatment). This included the methodology reported above, but also the dialysis of all the obtained lipoprotein fractions (320 samples) in order to deplete them of the salts used to obtain the specific density required in each step and the quantification of the protein content in each lipoprotein sample.

Protein content in the lipoprotein fractions of all patients was determined by the colorimetric protein assay kit BCA (Pierce, Thermo Fischer Scientific, Waltham, MA, USA), using a standard BSA calibration curve for quantification. Afterwards samples were adjusted to 100 µg/mL, an aliquot left protected from light at 4 °C for the functional analysis, and a second aliquot frozen at -80°C for analysis of lipid and protein patterns.

Standard lipid testing, represented by plasma levels of total cholesterol, LDL-C, HDL-C, and triglycerides, is a well-established platform for CVD risk prediction and management. However, it is known that lipoprotein particles are heterogeneous in size, density, charge, core lipid composition, specific apolipoproteins, and especially in their functionality. Therefore, specific lipoprotein protein/lipid composition tests and analysis of lipoprotein-fraction (i.e. HDL, LDL) activity have been proposed towards improving assessment of CVD risk and guiding lipid modifying therapies<sup>8</sup>.

Thus, **the third week** of my internship was devoted to performing functional assays for determining LDL susceptibility to oxidation, and the capacity of HDL to prevent LDL-oxidation.

Susceptibility of LDL to oxidation was assessed *in vitro* by determining the formation of conjugated dienes when LDL is exposed to copper-induced oxidation. Briefly, freshly prepared LDL samples adjusted to 100 µg/mL were analyzed after incubation with a copper (II) sulfate (CuSO<sub>4</sub>·5H<sub>2</sub>O) solution at a final concentration of 5 µM. The change of absorbance was determined

during 2.5 h at 37 °C using a SpectraMax 190 Microplate reader (Molecular Devices, Philadelphia, PA, USA) by continuously monitoring the formation of conjugated dienes, a product of lipid peroxidation, with absorbance peak at 234 nm. The time-course of the oxidation kinetics showed three consecutive phases, a lag-phase during which the diene absorption increases only weakly, a propagation phase with a rapid increase of the diene absorption and a decomposition phase. The determination of the lag-phase proved to be a convenient and objective procedure to determine the susceptibility of LDL towards oxidation.

The antioxidant potential of HDL was assessed by performing the total radical-trapping antioxidative potential (TRAP) test, a method based on the capability of HDL to prevent LDL oxidation. Briefly, HDL (patient samples before and after rosuvastatin treatment), and LDL lipoproteins (isolated from a pool of plasma obtained from normolipemic subjects) were adjusted to 100 µg protein/mL.

LDL derived from the control plasma pool was incubated with CuSO<sub>4</sub>·5H<sub>2</sub>O (final concentration of 20 µM) in the absence/presence of HDL from each individual subject (before and after rosuvastatin treatment) for 4h (37°C). Afterward, oxidation was stopped with EDTA and samples incubated with DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) in order to detect the oxidation level. DCFH-DA is a fluorogenic dye, that in the presence of esterases, is deacetylated to a non-fluorescent compound, which is later oxidized by the reactive oxygen species present in the samples, to DCF (2',7'-dichlorofluorescein). The intensity of DCF fluorescence was determined with a Typhoon FLA9500 (GE Healthcare, Chicago, IL, USA) set at  $\lambda_{ex} = 500$  nm and  $\lambda_{em} = 520$  nm. Final fluorescence measurements were obtained as a percentage of oxidized LDL generated in the presence of HDL, relative to the oxidation level when LDL was incubated in the absence of HDL.

During the end of the third and the fourth week of my stay, it was planned to perform analysis of HDL and LDL lipid patterns by thin layer chromatography, and determine the LDL and HDL protein patterns by 2D-electrophoresis and mass spectrometry (MS/MS), in order to identify changes in lipid and protein composition of both lipoprotein fractions in relation to the clinical characteristics of the patients and the response to rosuvastatin treatment. Unfortunately, due to the strict restrictions existing in Spain due to the increase of COVID19, this part of the study could not be carried out as planned, but will be performed once the working conditions are normalized.

As a contingency plan, during the fourth week of my stay in Barcelona, I have started the statistical analysis of the data, in order to relate the functional LDL and HDL variables studied with the patients' clinical characteristics, biochemical variables and their response to rosuvastatin treatment.

The results of the study are expected to be published in a high rank journal in the field. The support of the ESC, through the Mobility Travel Grant given by the Working Group on Coronary Pathophysiology & Microcirculation, will be acknowledged.

Sincerely,

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