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FINAL REPORT

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Attending Prof. Culmsee's laboratory has given me the opportunity to learn several techniques assaying mitochondrial dysfunction, that I have started to apply on my models of atherosclerosis. In cardiovascular disease there is a progressive decline of the mitochondrial function characterized by abnormalities in the respiratory chain and ATP synthesis, increased oxidative stress and loss of the structural integrity of mitochondria. By using the techniques I have handled in Marburg, I am currently conducting experiments trying to understand whether silencing of PCSK9 in liver cells and adipocytes is related to changes in mitochondria functionality.

Thanks to the contribution of IAS fellowship, I have had also the opportunity to publish one manuscript:

1. Macchi C, Sirtori CR, Corsini A, Santos RD, Watts GF, Ruscica M. A new dawn for managing dyslipidemias: The era of RNA-based therapies. Pharmacol Res. 2019 Dec;150:104413. doi: 10.1016/j.phrs.2019.104413.
In the acknowledgement section has been reported "CM was recipient of a scholarship generously offered by the International Atherosclerosis Society (IAS)."

Below described the techniques I have learnt thanks to the sponsorship of IAS.

Lipid peroxidation
Lipid peroxidation can be quantified using BODIPY 581/591 C11 (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) as a sensor for the oxidation of lipids and membranes. Specifically, oxidation of the polyunsaturated butadienyl portion of the dye results in a shift of the fluorescence emission peak from ~590 nm to ~510 nm, that can be detected by flow cytometry.

Mitochondrial reactive oxygen species (ROS) formation
Mitochondrial superoxide production can be visualized by using the MitoSOX staining in fluorescence microscopy and by subsequent flow cytometry analysis. MitoSOX™ Red reagent permeates live cells where it selectively targets mitochondria. It is rapidly oxidized by superoxide, exhibiting red fluorescence, but not by other ROS and reactive nitrogen species. The increased red fluorescence, due to the formation of mitochondrial ROS, can be detected by flow cytometry. MitoSOX™ Red reagent may also provide a valuable tool in the discovery of agents that modulate oxidative stress in various pathologies.

Mitochondrial membrane potential
Mitochondrial membrane potential can be determined using the MitoPT TMRE staining followed by flow cytometry analysis. TMRE is a cell permeant, positively charged, red-orange dye that accumulates in active mitochondria due to their relative negative charge. Upon loss of the mitochondrial membrane integrity and, thus, membrane potential, TMRE fails to reach mitochondria and the consequent loss of TMRE fluorescence can be detected by flow cytometry.

Mitochondrial morphology
MitoTracker® Deep Red FM can be used to analyze mitochondrial mass, length and shape. In particular, cells can be categorized according to the morphology of their mitochondria. Category I is defined by elongated and equally distributed mitochondria, organized in a tubular network, category II is characterized by partially fragmented mitochondria, which are still equally distributed throughout the cytosol, whereas category III is represented by fully fragmented mitochondria, that surround the nucleus.

ATP measurements
ATP levels can be detected by a bioluminescent measurement which can also allow to analyze the cellular proliferation and cytotoxicity. In detail, this assay is based on the enzyme luciferase, which catalyses the formation of light from ATP and luciferin. The intensity of the emitted light is linearly related to the ATP concentration and is measured by a luminescence detection.

Seahorse measurements
Since mitochondria are key providers of energy to the cell in the form of adenosine triphosphate (ATP) through OXPHOS, consumption rate (OCR), a measurement of mitochondrial respiration, and extracellular acidification rate (ECAR) as measure of glycolysis, can be determined in real-time using the XF Extracellular Flux Analyzer, in the presence of specific mitochondrial activators and inhibitors. Specifically, ATP synthase blocker (oligomycin) is used to determine proton leak, while mitochondrial un-coupler carbonyl cyanide 4-[trifluoromethoxy] phenylhydrazone (FCCP) to measure maximum respiratory function (maximal OCR). Reserve capacity is calculated as a maximal OCR minus the basal respiration. The Inhibitor of complex I (rotenone) and a blocker of complex III (antimycin A) are injected to completely abolish the mitochondrial respiration, to confirm that any observed changes in respiration is mediated by mitochondria.

During my stay at the Prof. Culmsee's laboratory I had the opportunity to investigate the role of an actin binding protein in the crosstalk between mitochondria and actin cytoskeleton. To this purpose, I used, as an *in vitro* model, mouse embryonic fibroblasts (MEFs) isolated from animal specifically knockout for the considered protein and the corresponding wild-type littermates. To elucidate the relevance of the actin binding protein on mitochondrial morphology and function I took advantage of the techniques described above. The competencies acquired, thanks to this fellowship, will allow to implement my research program by using the learnt techniques to investigate the role of PCSK9 in hepatocytes and adipocytes. Moreover, I could also expand my study on atherosclerosis by isolating MEFs from PCSK9$^{-/-}$, apoE$^{-/-}$ and LDLR$^{-/-}$ mice to study the effects of these deletions on mitochondrial activity.