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# Comparative proteomic analysis of membrane microdomains isolated from two hyperlipidemic animal models

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ABSTRACT

Hyperlipidemia is a common risk factor for the initiation and progression of cardiovascular diseases, affecting complex signaling pathways and leading to a fatal outcome in the most severe events. It has been proven that the non-ionic detergent insoluble membrane microdomains are enriched in signaling molecules, taking part in various essential physiological but also pathological processes. The aim of the present study was to demonstrate the comparable alteration of membrane signaling pathways produced by either genetically or diet induced hyperlipidemic stress.

Using two established hyperlipidemic laboratory animal models, the ApoE deficient mouse and the diet induced hyperlipemic Golden Syrian hamster, we have analyzed the proteomic profile of detergent resistant membrane microdomains in hyperlipidemic and statin treatment conditions versus the appropriate control states. Employing latest generation liquid chromatographic and mass spectrometric approaches followed by specialized software analysis allowed us to discover with high degree of confidence protein molecules' inter-relation maps affected by hyperlipidemia and statin treatment such as leukocyte transendothelial migration, tight junctions, phagosomal signaling, common to both types of organisms. However, the different methods to induce the high fat stress revealed uniquely altered signaling pathways in *antigen processing and presentation*, *citrate cycle*, *extracellular matrix-receptor interaction*, *adherence junction and focal adhesion* in one or the other organism.



## 1. Introduction

Atherosclerosis is a disease mainly of the large and medium caliber arteries with severe clinical manifestations and often tragic end (cardiac ischemia, lower limb gangrene, ischemic encephalopathy, myocardial infarction, cerebral infarction). Hyperlipidemia is a major risk factor in atherosclerosis, statin treatment being the most common measure to counteract the increased circulating lipid levels [1]. Recently published data supports the concept that atherosclerosis is a systemic inflammatory vascular disease with multiple dis-functionalities at cellular and molecular level [2,3]. The inflammatory mediators and cytokines/chemokines released in the atherosclerotic prone area could affect the homeostasis of both macro- and micro-vascular system in the whole body leading towards high risk vulnerable patients. In this context, pulmonary endothelium is directly involved in a number of vital functions of the body (solute exchange, regulation of vascular tone, vasculogenesis) and although it does not normally develop atherosclerotic plaques, it can be activated by pro-atherogenic stress factors, such as hyperlipidemic diet [4], hypertension [5], deregulated production of nitric oxide [6], etc., with implications in altering signaling pathways in the cellular components of the vascular wall.

The complex structure of the biological membranes maintain cellular homeostasis by efficient mechanisms integrated in a structural and functional barrier. Membrane microdomains represent dynamic membrane nano-assemblies with a special protein and lipid content involved in transport, cholesterol homeostasis, signaling, etc. [7,8]. This particular composition determines their characteristic of being insoluble in nonionic detergents and flotation in a density gradient following ultracentrifugation at 200,000xg [9]. Their protein profile is enriched in signaling proteins, which suggests their active involvement in not only physiological but also pathological molecular processes.

Golden Syrian hamsters (*Mesocricetus auratus*) develop atherosclerotic plaques under a high fat rich diet [10]. It has been demonstrated that administration of this diet for 8–12 weeks results in elevated levels of plasma cholesterol and triglycerides. Unlike other small laboratory animals, but similar to humans, the intake of high contents of lipids by Golden Syrian hamsters is enough to generate atherosclerotic lesions at the level of cardiac valves [3,4].

Genetically modified ApoE deficient mice (*Mus musculus*) spontaneously develop atherosclerotic lesions even on a low fat diet [11–13]. This animal model exhibits extremely high levels of plasma cholesterol and

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more advanced lesions on the aorta when compared to the classical fatty streaks in other murine races.

The present workflow took advantage of the refined method of membrane microdomains isolation methodology and the high performance liquid chromatography - mass spectrometry based proteomic techniques to investigate the alteration of the molecular mechanisms in pulmonary endothelium detergent resistant membrane microdomains following a hyperlipidemic stress applied either genetically or diet induced on these two laboratory small animal models: the ApoE deficient mouse and Golden Syrian hamster fed with a hyperlipidemic diet. The statin therapy, highly relevant in the context of human disease similarities was also evaluated. In the end, the data analysis, unequivocally, identified and quantified with high accuracy and significance sets of proteins implicated in various signaling pathways that are commonly but also uniquely over-represented in the two experimental conditions, suggesting similar or distinct molecular mechanisms by which hyperlipidemia acts on the detergent resistant membrane microdomains proteome.

## 2. Materials and methods

### 2.1. Reagents

All reagents were of electrophoresis, liquid chromatography or mass spectrometry grade. Sodium fluvastatin was purchased from Novartis (Basel, Switzerland); MES [2-(*N*-morpholino)ethanesulfonic acid], sodium chloride, urea, sodium deoxycholate (DOC), trizma hydrochloride, DL-dithiothreitol (DTT), iodoacetamide (IAA), *N*-acetyl-L-cysteine (NAC), ammonium bicarbonate, bicinchonic acid, Bradford reagent and all solvents were provided by Sigma-Aldrich (Missouri, USA). Sequencing grade modified trypsin was offered by Promega (Wisconsin, USA). Protease inhibitor Complete cocktail was purchased from Roche (Indiana, USA). C18 solid phase extraction columns were acquired from Waters (Massachusetts, USA).

### 2.2. Animal models

Healthy 6 weeks male laboratory mice (*M. musculus*) and 3 months male Golden Syrian hamsters (*M. auratus*) were used in the study. The mice lot comprised a Black C57 control group (C, $n = 3$) fed four weeks with standard diet, a group of transgenic hyperlipidemic ApoE deficient mice ($A_m$, $n = 3$), that received for the same period of time a high fat diet (1% cholesterol and 15% butter) and a statin treated group of ApoE deficient mice ($At_m$, $n = 3$) that were transferred to standard diet together with oral gavages of fluvastatin sodium (10 mg/kg body/day) for two weeks, after the four weeks of high fat intake. The hamsters lot was divided into 3 categories: a control one (N, $n = 3$), which received a normal diet for 6 months, a hyperlipidemic one ($A_h$, $n = 5$), fed a high fat diet (3% cholesterol and 15% butter) for 6 months and a statin treated hyperlipidemic group ($At_h$, $n = 3$) that received a hyperlipidemic diet for 3 months, after which it was changed with a standard one, together with the administration of sodium fluvastatin (10 mg/kg body/day).

The animals were kept in the experimental modeling husbandry facility under 12 h light/dark cycles with full time free access to food and water. All animal experiments were conducted in accordance with "International Guiding Principles for Biomedical Research Involving Animals" (Council for the International Organizations of Medical Sciences, December 2012), the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and Romanian Law no. 471/2002.

### 2.3. Detergent resistant membrane microdomains isolation

Detergent resistant membrane (DRM) microdomains were prepared as previously mentioned [9]. Briefly, 200 mg of lung tissue fragments were homogenized at 4 °C in 1.8 ml MES-buffered saline (MBS), pH 6.5, which contained 25 mM MES, 0.15 M NaCl and 1% Triton X-100 using a rotor-stator mechanical device. The resulting homogenate was corrected to 40% sucrose with 2 ml of 80% sucrose in MBS and placed on the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was formed by overlaying 4 ml of 30% and 4 ml of 5% sucrose in MBS. The sucrose gradient tubes were placed in the SW-41 rotor and centrifuged at 200,000xg, for 19 h at 4 °C, using the Optima LE-80 system (Beckman-Coulter, Fullerton, USA). 12 fractions were harvested from top to bottom, for afterwards experiments. Protein quantification was performed using bicinchoninic acid and cholesterol level determination was realized using the CHOD-PAP method (cholesterol assay kit, DIALAB GMBH, Neudorf, Austria). For each individual biological replicate, the two fractions (4 and 5) enriched in protein and cholesterol, with high angiotensin converting enzyme (ACE) activity (data not shown), were combined and diluted (5 times) in MBS buffer before ultracentrifugation (4 h, 200,000xg at 4 °C). The resulting supernatant was removed and the pellet was stored at −80 °C until further analysis.

### 2.4. Sample preparation for mass spectrometric analysis

Solubilization and denaturation were performed in a buffer containing 8 M urea, 1% sodium deoxycholate (DOC) and 0.1% Tris-HCl (pH 8.8) through powerful vortexing on ice. The protein quantification was performed using the Bradford reagent and a bovine serum albumin 5 points (0.1–1 μg/μl) standard curve. Afterwards, the protein samples were purified by methanol/chloroform/ water (4:2:4) precipitation, followed by reduction (with buffer containing 8 M urea, 0.1 M Tris-HCl, 0.1 mM EDTA and 20 mM DTT) and alkylation of the cysteine residues (using 80 mM IAA in 0.1 M Tris-HCl and 0.1 mM EDTA buffer). The iodoacetamide excess was quenched with 80 mM NAC in 0.1 M Tris-HCl and 0.1 mM EDTA buffer. Prior to the digestion process, the sample buffer was diluted up to 1 M urea using 50 mM ammonium bicarbonate (pH 8.8) and DOC was added up to 1% final concentration.

Proteolysis was performed for 14 h, at 37 °C, with stirring, at 1:20 enzyme to substrate quantity ratio, using sequencing grade modified trypsin. The resulting peptide mixtures were acidified to pH 2–3 with formic acid for trypsin activity inhibition and DOC precipitation which was discarded following a 20 min. 20,000 ×*g* centrifugation. Desalting was conducted using Sep. Pak C18 columns. The purified peptides were eluted using 0.1% formic acid in 80% acetonitrile. The peptides were dried using the Concentrator plus system (Eppendorf, Hamburg, Germany) and stored at −80 °C until LC-MS analysis. Prior to chromatographic separation, the peptides were re-suspended in 0.1% formic acid and 5% acetonitrile solution to final concentration of 0.5 μg/μl, using an ultrasonication bath.

### 2.5. Liquid chromatography-mass spectrometry analysis

LC-MS/MS experiments were performed using the Ultimate 3000 RSLC nano system (Dionex, California, USA) coupled to the LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Scientific, California, USA). For each analysis, the sample (1 μl) was loaded in triplicate, into an Acclaim PepMap 2 cm × 75 μm i.d., C18, 3 μm, 100 A trap column (Dionex) connected to the Acclaim PepMap RSLC 15 cm × 75 μm i.d., C18, 2 μm, 100 A analytical column (Dionex). Solvent A was LC-MS grade water with 0.1% (v/v) formic acid, and solvent B was represented by LC-MS grade acetonitrile with 0.1% (v/v) formic acid. Peptides were eluted with a gradient of 2–35% solvent B over 48 min (70 min total chromatographic method and MS acquisition) at 300 nl/min flow rate. Dynamic nano-electrospray source housing was utilized with 12 cm length, 360 μm outer diameter, 20 μm inner diameter and 10 μm tip inner diameter uncoated SilicaTips. 1500 V of liquid junction voltage and 250 °C capillary temperature were used. The MS was operated in a top 6 data-dependent configuration at 60 k resolving power for full scan, with monoisotopic precursor selection enabled and mass