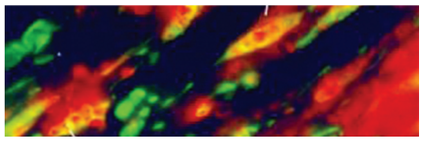
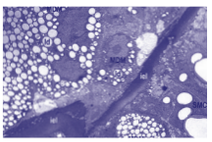
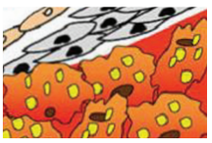


DEPARTMENT OF LIPIDOMICS



LABORATORY OF LIPOPROTEINS AND ATHEROSCLEROSIS



MOLECULAR BIOLOGY OF LIPOPROTEINS LABORATORY

***Anca V. Sima**, PhD*
HEAD OF DEPARTMENT

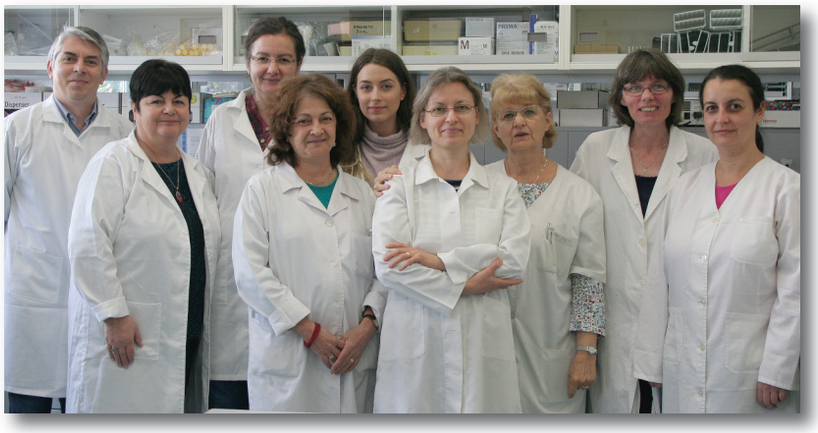
STAFF

***Camelia S. Stancu**, PhD*
HEAD OF LIPOPROTEINS AND ATHEROSCLEROSIS LABORATORY
***Loredan S. Niculescu**, PhD*
HEAD OF MOLECULAR BIOLOGY OF LIPOPROTEINS LABORATORY
***Laura Toma**, PhD*
***Gabriela M. Sanda**, PhD*
***Mariana Deleanu**, Chemical engineer*
***Teodora Barbălată**, PhD student*
***Daniela Rogoz**, Technical assistant*
***Cristina Dobre**, Technical assistant*

FORMER RESEARCH STAFF:

Eliza Vasile, Doina Filip, Elena Constantinescu, Gabriel Popescu, Rozalia Mora, Alexandru Bulla, Anca Dobrian, Daniela Tîrziu, Silviu Faitar, Oana Sorop, Cosmin Catana-Negreanu, Marius Robciuc.

CORE LABORATORY UNIT:
Liquid and gas chromatography
responsible:
Mariana Deleanu



DEPARTMENT OF LIPIDOMICS

AncaV. Sima, *PhD*

Head of Department
E-mail: *anca.sima@icbp.ro*

Major position/appointments and professional training

- Member of the Romanian Academy
- Scientific Secretary of ICBP-NS
- PhD Advisor in Biology, Advisor for graduate and master programs
- President of the Biology and Biochemistry Commission of the Romanian Council for Attestation of University Titles, Diplomas and Certificates
- Executive Director of the Advanced Study Course of Cellular and Molecular Medicine
- Expert evaluator of national and international grants
- Member of the Editorial Board of Scientific Reports (Nature group)

MAJOR RESEARCH INTERESTS

● **Lipid metabolism in health and disease**: dysregulation of lipid metabolism in atherosclerosis, diabetes, metabolic syndrome and obesity, cellular and molecular biology of lipoproteins (Lp), transport of Lp across the vascular endothelium, interaction of Lp with the cells of the arterial wall, in vivo and in vitro modification of low density Lp (LDL), dysfunctional high density Lp (HDL), entero-hepatic metabolism of Lp, molecular biology of proteins involved in cellular cholesterol efflux, implication in atheroma formation.

● **Cellular biology and biochemistry of blood vessels**: heart and cardiac valves in pathological conditions, biochemical and biophysical modifications in atherogenesis, diabetes and obesity, experimental animal models of atherosclerosis and/or diabetes.

● **Novel biomarkers for cardiovasculardisease**: dysfunctional Lp, oxidized lipids, Lp-associated enzymes, inflammatory mediators.

● **Genetic and epigenetic mechanisms of atherosclerosis and/or diabetes**: gene polymorphisms, functional analysis of RNA-related epigenetic markers (microRNAs, lncRNAs), identification and validation of specific microRNAs target genes, gene editing of lipid-related proteins to improve cardiovascular diseases.

● **Pharmacologic attempts to arrest or reverse cardiovascular diseases**: pleiotropic properties of anti-atherosclerotic drugs (statins, calcium channel blockers - amlodipine), biologically active natural compounds (probiotics, caffeic acid, ginger extract).

PUBLICATIONS

Over 90 original articles (>2,000 citations) were published in Web of Sciences Core Collection journals and 11 book chapters between 1979-2019 by researchers of the Lipidomics Department.

● 2 articles with over 200 citations, 5 articles with over 100 citations

● H index of team members: 7– 20

SELECTED NEW FINDINGS OF THE DEPARTMENT

• HDL from acute coronary syndrome (ACS) patients become pro-inflammatory and correlate with a panel of plasma parameters (apoC-III, MPO, oxidized-apoA-I, ceruloplasmin, and PON1), and together can discriminate between this group and stable angina CAD patients.

• A new mechanism by which hyperlipidemia induces dysfunctional HDL production in the small intestine and liver of hyperlipidemic hamsters: high fat diet induces endoplasmic reticulum stress and decreased expression of LXRβ and PPARγ (regulators of HDL apolipoproteins and enzymes production).

• A panel of circulating miRNAs was identified in sera from hyperlipidemic and/or hyperglycemic subjects, correlated with increased lipids and inflammatory markers.

• Two circulating miRNAs, miR-486 and miR-92a, together with dysfunctional HDL can be used as an additional statistical tool to designate vulnerable CAD patients.

• A preferential distribution of miR-486 and miR-92a in the HDL subpopulations (HDL2, HDL3) can discriminate between ACS and stable CAD patients.

• Hyperglycemia determines increased specific microRNAs levels in sera and HDL of ACS patients and stimulates microRNAs production in human macrophages.

• Inhibition of miR-486 and miR-92a decreases liver and plasma cholesterol levels by modulating lipid-related genes in hyperlipidemic hamsters.

• Probiotics exert lipid-lowering effects based on the entero-hepatic regulation of cholesterol metabolism, increased HDL apolipoproteins and enzymes synthesis, decreased oxidative stress and lipid deposits in aortic valves, all mediated by upregulation of nuclear receptors PPARγ/RXR and LXRs.

• Probiotics administration decrease serum and hepatic expression of miR-223, miR-122a, miR-92a and miR-486, the miRNAs production proteins (Dicer, DGCR8) in the HL livers, and decrease lipid deposits in the aortic valves.

• Ginger extract exerts anti-inflammatory action by decreasing ninjurin 1 (Ninj-1), TNFα receptor 1 and NADPH oxidase subunits expression and increasing sRAGE levels in the culture media of TNFα-exposed human endothelial cells.

• The simultaneous administration of ginger extract with the high-fat diet induces in hamsters' livers the inhibition of ERS and the increase of LXRα/β and PPARγ protein expression, leading to increased synthesis of ABCG5/G8 and CYP7A1 proteins and the decrease of accumulated cholesterol.

• Ginger extract reduces the oxidized-apoAI levels (induced by the high-fat diet) by diminishing the MPO/PON1 ratio; these effects are associated with the retrieval of SIRT1-LXRα/β-PPARγ pathway, that further adjust the levels of MPO, PON1 and ABC transporters in hamster's small intestine.

• Simvastatin inhibits transcytosis of LDL in hyperlipemia reducing plaque progression.

• Simvastatin and Amlodipin increase the sera antioxidant potential in patients with stable angina.

• Atorvastatin downregulates NADPH oxidase activity, and decreases NOX1 and p22phox gene expression in human aortic smooth muscle cells exposed to glycated LDL.

• PPAR agonists decrease plaque vulnerability through modulation of MMP-2 activity.

• Anti-oxidant potential of felodipine is higher than that of amlodipine.

PREVIOUS PROJECTS

1. TRANSCYTOSIS OF LDL THROUGH THE VASCULAR ENDOTHELIUM

All plasma macromolecules cross the vascular endothelium to reach the interstitial fluid and subendothelial cells. Results obtained by Nicolae Simionescu, Maya Simionescu and George E. Palade revealed that in endothelial cells (EC) the transport of macromolecules is accomplished via vesicles, channels, and fenestrae and depends both on the chemistry of plasma macromolecules and on the biochemical makeup of the EC plasmalemma. Based on accumulated data, Professor Nicolae Simionescu introduced the concept and coined the name "transcytosis" (transport across the cell) as a basic cellular process and identified its mechanisms - fluid-phase-, adsorptive- and receptor-mediated transcytosis of plasma proteins (*Simionescu* N., 1989).

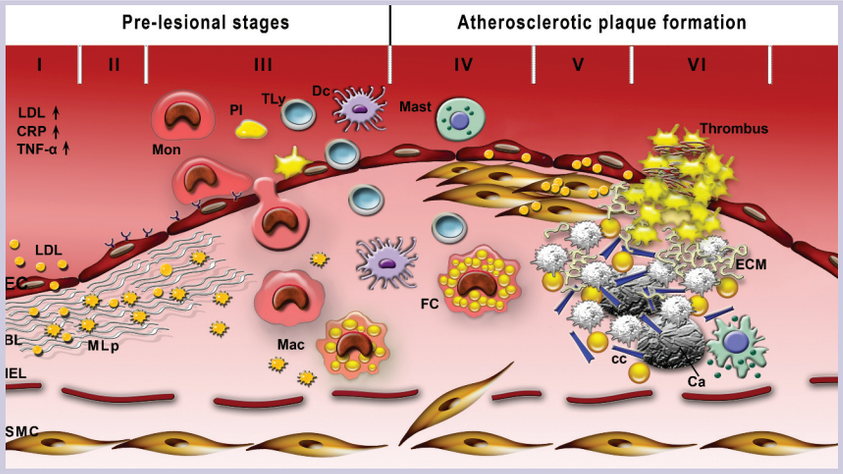
The new findings were published in:

• Transcytosis and endocytosis of LDL through endothelium in arteries and lung capillaries *(Vasile E. et al., J.Cell.Biol. 1983;Nistor-Sima A. and M. Simionescu, Am. J. Resp. Diseases 1986*).

2.PATHOBIOCHEMICAL EVENTS OF ATHEROSCLEROSIS - AN ORIGINAL MODEL, THE HYPERLIPEMIC HAMSTER

• pathobiochemical events occurring at the inception of atherosclerosis
• lesional stages of atherosclerosis

Atherosclerosis is the main cause of morbidity and mortality both in industrialized countries and in Romania. An important step in the study of a disease is finding a suitable animal model. We introduced as experimental model to study atherogenesis the Golden Syrian hamster fed a fat-rich diet, which develops hyperlipidemia and atherosclerotic plaques similar in many respects to human atheroma *(Nistor-SimaA. et al., Atherosclerosis 1987; SimaA. et al.,J.Submicrosc.Cytol.Pathol. 1990)*.
Understanding of the prelesional stage is crucial because it is the critical point at which therapeutic interventions to retard or regress the evolution of the atherosclerotic plaque can be most efficient. Based on a large number of experiments on hyperlipemic rabbits and hamsters, prelesional and lesional stages of atherosclerosis were identified and the sequence of events occurring in each stage were documented *(Simionescu N. et al., Am. J. Pathol. 1986; Mora R. et al., J. Lipid Res. 1986; Nistor-Sima A. et al., Atherosclerosis 1987; Filip D. et al., Atherosclerosis 1987).*



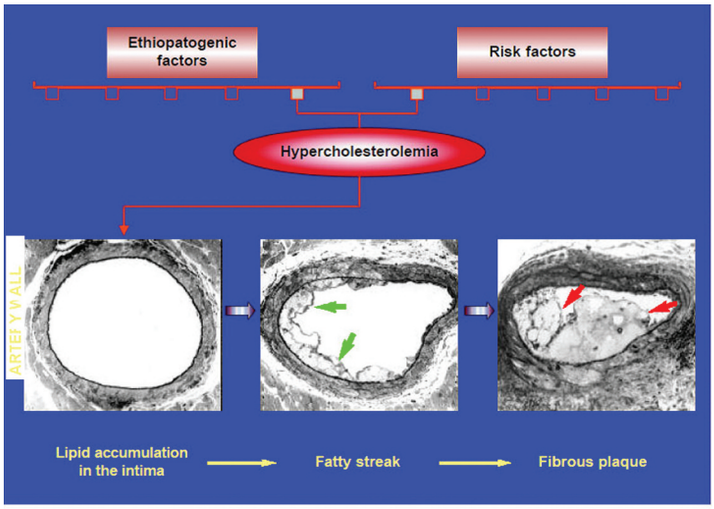
Diagrammatic representation of the atheroma formation and the arbitrarily delineated consecutive stages

Diagrammatic representation of the atheroma formation and the arbitrarily delineated consecutive stages.

Stage I*, initiation of the plaque, characterized by modulation of endothelial cell (EC) constitutive functions due to increased concentrations of plasma low density lipoproteins (LDL), C-reactive Protein (CRP), or tumor necrosis factor (TNF-α). In* ***stage II****, LDL trapped within the intima undergo alterations (oxidation, glycation), turning into modified lipoproteins (MLp), inducing EC dysfunction.* In ***stage III*** *a robust inflammatory reaction determines monocytes (Mon) assisted by platelets (Pl), T lymphocytes (TLy) and dendritic cells (Dc) to adhere and then enter the arterial intima. Mon become activated macrophages (Mac) that take up MLp and turn into macrophage derived-foam cells (FC) and form fatty streaks. In* ***stage IV****, smooth muscle cells (SMC) glide from the media into the intima, forming the fibrous cap. The fibro-lipid plaque comprising SMC-, Mac- and EC-derived foam cells defines* ***stage V****. Extracellular matrix (ECM), cholesterol crystals (cc) and large calcification cores (Ca) are formed. The complicated plaque becomes vulnerable* ***(stage VI)****, exhibiting fibrous cap thinning and rupture, EC damage and death and the subsequent exposure of ECM, Pl adherence and thrombus formation (Thrombus) (Simionescu M., A.V.Sima. In: "Inflammation and Atherosclerosis", Springer-Verlag/Wien, 2012).*

The new findings were:

In the prelesional stage the initial modifications that appear in aortic lesion prone areas and heart valves (prior to monocyte adhesion) are:



- Glycocalix disappearance and modification of the net electrical surface charge of the endothelium.
- Increased transcytosis of plasma lipoproteins (Lp) through the endothelium.
- Enhanced synthesis of a hyperplasic basal lamina of the endothelium.
- Lp transcytosed from plasma are retained and accumulate within the subendothelium, in the meshes of the multilayered basal lamina, as modified and reassembled Lp (MRL); Lp deposition in the arterial intima and the cardiac valves.

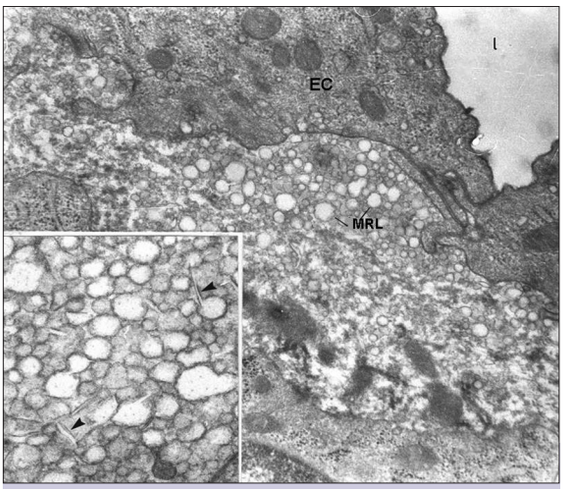
The lesional stages of atherosclerosis are characterized by:

- *Monocytes* diapedesis that display the general features of an inflammatory process. Within the subendothelium, monocytes become activated macrophages that take up MRL, turning into macrophage-derived foam cells.
- *Smooth muscle cells* (SMC) migrate into the intima, where they form either the

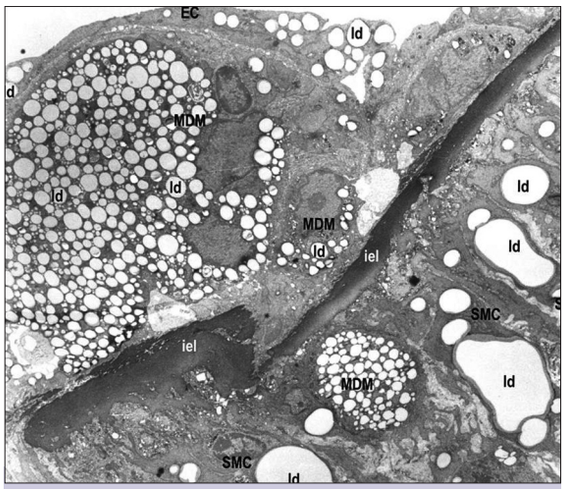
fibrous cap or become smooth muscle-derived-foam cells.

- *Endothelial cells* (EC) maintain their integrity, but they become dysfunctional and alter the vascular wall and plasma homeostasis. In advanced plaques, EC turn into foam cells and calcification centers appear within the vessel wall.

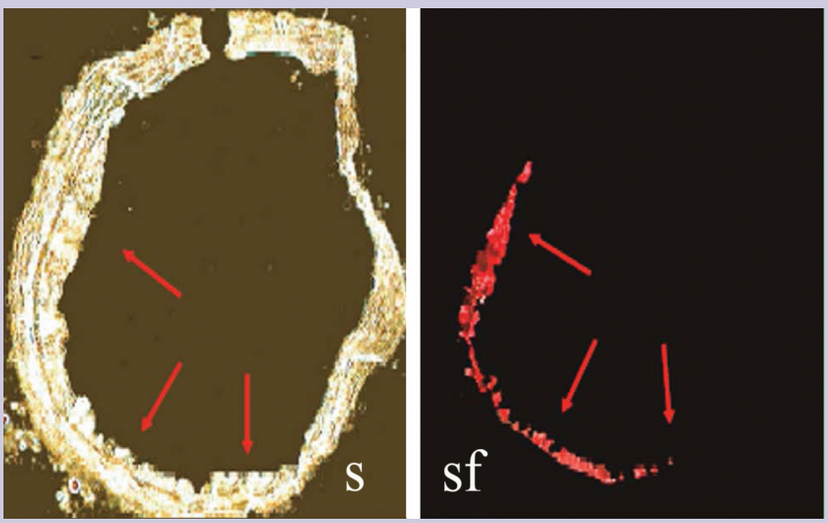
- *The antioxidant potential* decreases in sera from hyperlipidemic compared to normal hamsters.



Accumulation of modified and reassembled lipoproteins (MRL) under the endothelial cells (EC) in an arterial lesion-prone area of a hyperlipidemic hamster
(Nistor-Sima A., Atherosclerosis 1987).



Late coronary atheroma in a hyperlipidemic hamster. The endothelium (EC) rich in lipid droplets (ld) overlays lipid-loaded monocyte derived macrophages (MDM) and smooth muscle cells (SMC)
(Sima A., J. Cell. Mol. Med.2002).



Fluorescent LDL accumulated in the aortic intima of a hyperlipidemic hamster
(Stancu C., L. Toma and A. Sima, Cell Tissue Res. 2012).

3. PATHOBIOCHEMISTRY OF COMBINED DIABETES AND ATHEROSCLEROSIS

Studies in an original model of simultaneously hyperlipidemic-diabetic hamster

A complex biochemical and ultrastructural study was initiated by Prof. Maya Simionescu, in collaboration with the laboratory for Vascular Dysfunction in Diabetes and the Lipoproteins laboratory, in a simultaneous hyperlipemic and hyperglycemic hamster.

The new findings were:

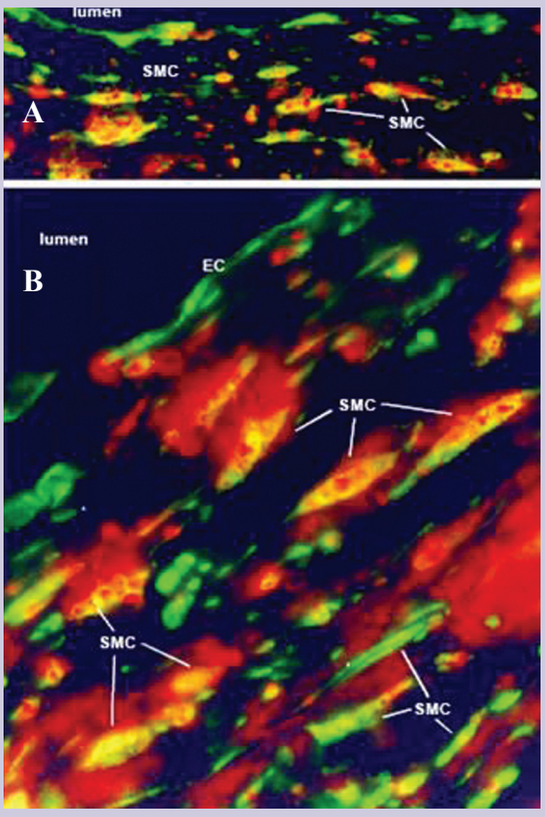
Structural changes occurring in cardiac valves, coronary arteries and aorta revealed that the humoral modifications coexisted with micro- and macroangiopathic lesions characteristic to both diseases; accelerated formation of atherosclerotic plaques occurred in lesion prone areas (*Simionescu M., et al., Am. J. Pathol. 1996)*.

Modified LDL (oxidized and/or glycated) and AGE-proteins are present in the intima of hamsters coronary arteries and heart valves; identification by immunohistochemical methods *(Sima A. et al., Lab. Invest. 1997*).

Studies in human atheroma: immuno-detection of oxidized and glycated LDL

The new findings were:

Immunolocalization of LDL, LDL oxidation products, 4-hydroxynonenal (HNE) and advanced glycation end-products (AGE) in the atherosclerotic plaques from diabetic CAD patients. HNE and AGE colocalized on cryosections from human atheroma, being present both intracellularly, in macrophage-derived foam cells from the shoulder areas and in the smooth muscle cells of the fibrous cap, and extracellularly, in lipid deposits near the internal elastic lamina.



Immunolocalization on cryosections from human coronary atheroma of:

A*. 4-hydroxy-nonenal (HNE) (green fluorescence) and* ***B****. advanced glycation end products (AGE) (green fluorescence) in endothelial cells (EC) and in smooth muscle cells (SMC) from the fibrous cap. The red fluorescence represents the Oil Red-O staining of the lipid deposits, the yellow results from the superposition of the green and red ones*
(Sima A. and C. Stancu, J. Cell. Mol. Med. 2002).

4. MOLECULAR MECHANISMS ASSOCIATED WITH MODIFIED LP IN THE CELLS OF THE HUMAN ARTERIAL WALL

Endoplasmic reticulum stress and oxidative stress in oxLDL-exposed macrophages. OxLDL alter the proper function of the endoplasmic reticulum, inducing ER stress (ERS), which consequently activates inflammatory pathways in macrophages. Matrix metalloproteinase-9 (MMP-9) is the main protease acting on the degradation of the extracellular matrix and the ensuing destabilization of the atherosclerotic plaque. We have investigated whether ERS induced by oxLDL in human macrophages is associated with the stimulation of MMP-9 expression and secretion (*Sanda G. et al., J. Cell. Biochem. 2017)*.

The new findings are:

• OxLDL induce the intracellular accumulation of 7-ketocholesterol in lipid-loaded macrophages, the most prominent oxysterol formed during the oxidative modification of LDL.
• OxLDL increase MMP-9 gene expression and pro-MMP-9 secretion in human lipid-loaded macrophages, through the activation of the PERK-eIF2α, a branch of the unfolded protein response (UPR).
• ROS induce MMP-9 secretion through the activation of ERS (IRE1α-XBP1 branch of UPR).

Modulation of cholesterol efflux by apoE secretion in lipid-loaded macrophages

We have investigated the signaling pathways involved in apoA-I and HDL-mediated regulation of cholesteryl ester transfer protein (CETP) and apoE secretion from lipid-loaded macrophages. We asked whether HDL may inhibit ERS in correlation with the secretion of apoE and CETP from lipid-loaded macrophages *(Niculescu L. et al., Biochem. Biophys. Res. Commun., 2011 and 2013)*.

The new findings are:

- Human apoA-I and HDL_3 stimulate the expression and secretion of CETP from lipid-loaded macrophages, mediated by PKA signaling pathway.
- A significant inhibition of apoE synthesis and secretion after siRNA-mediated CETP gene silencing in both non-loaded and lipid-loaded macrophages.
- A new molecular mechanism by which apoA-I induces CETP secretion from lipid-loaded macrophages involving both NF-κB and PKA signaling pathways.
- The addition of HDL_3 to the culture medium of tunicamycin-treated cells induced: (i) the reduction of ERS, expressed as decreased levels of eIF-2α and SAPK/JNK, and (ii) a partial recovery of the secreted apoE and CETP in lipid-loaded macrophages.

Effect of irreversibly glycated LDL in human endothelial and vascular smooth muscle cells

In diabetes, hyperglycemia and the associated formation of advanced glycation end-products (AGE) and AGE-modified low density lipoproteins (AGE-LDL) can directly affect the cells of the vascular wall. We hypothesized that AGE-LDL may act directly and induce oxidative and inflammatory alterations in human endothelial cells (HEC) and human vascular smooth muscle cells (hSMC), this effect being amplified by a high glucose content (*Toma L. et al., Biochem. Biophys. Res. Commun. 2009, Sima A. et al., J. Cell. Mol. Med. 2010)*.

The new findings were:

- AGE-LDL induce oxidative stress and a pro-inflammatory state in HEC. Both AGE-LDL and nLDL in the presence of high glucose amplify this effect, revealing a link between hyperlipidemia, diabetes and endothelial cell dysfunction.
- AGE-LDL activate hSMC (increasing

CD36, LRP1, RAGE), inducing a pro-oxidant state (activation of NADPHox), lipid accumulation and a pro-inflammatory state (MCP-1 synthesis). These results may partly explain the contribution of AGE-LDL and hSMC to the accelerated atherosclerosis in diabetes.

5. GENE POLYMORPHISM OF APO-LIPOPROTEINS - RISK FACTORS FOR SUBJECTS WITH METABOLIC SYNDROME

Apolipoprotein E and apolipoprotein A-V
Genetic variation at the APOE (on 19q13.2 chromosome) and APOA5 (proximal to A*POA1/C3/A4* gene cluster) are associated with dyslipidemia, a risk factor for atherosclerosis and diabetes. We determined the association of APOE genotypes and *APOA5* gene polymorphisms (-1,131T>C and c.56C>G) in 279 subjects with metabolic syndrome (MS).

The new findings were:

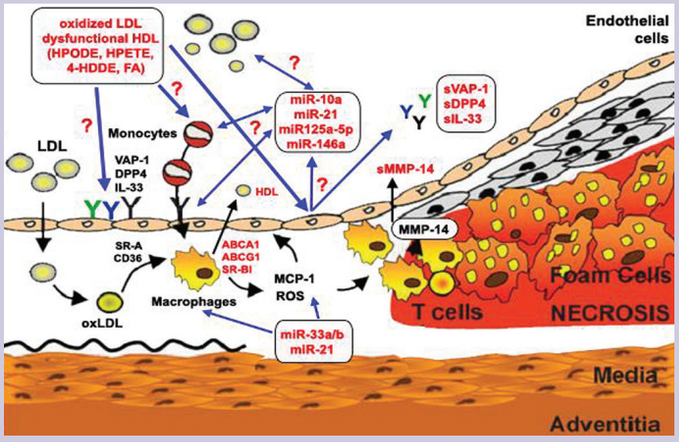
- Significant differences for apoE genotype frequencies between normal-weight and obese subjects in either MS or coronary heart disease (CHD) groups were found. The data indicate that apoE polymorphism can be considered a major, independent risk factor for MS (*Sima A. et al., Clin. Chem. Lab. Med., 2007*).
- APOA5 -1.131C variant is associated with increased plasma triglyceride levels in MS subjects. Our results reveal a correlation between APOA5 polymorphisms, plasma triglycerideand apoA-V levels in CHD patients only for the -1.131CC genotype (*Niculescu L.et al., Clin. Chem. Lab. Med., 2007; Niculescu L.et al., Biochem. Biophys. Res. Commun., 2010*).

CURRENT PROJECTS

1. NEW BIOMARKERS PREDICTIVE FOR THE EVOLUTION OF THE CORONARY ARTERY DISEASE

Our goal was to establish new predictive biomarkers for the risk of life-threatening events in coronary artery disease (CAD) patients. The originality of the project resided in the final design of a protocol using a group of biomarkers implicated in the three pathways of atherosclerosis (oxidation, inflammation and transcriptional regulation) that have the potential to predict CAD events and transfer the methodology from bench to the clinics. The evaluated inflammation markers of CAD were: VAP-1, IL-33, DPP4, MMP-14. Some epigenetic markers specific for CAD, such as microRNAs (miR-21, miR-33, miR-10a, miR-125a, miR-146a) were investigated.

Potential new biomarkers with prognostic values for CAD evolution. *Vascular adhesion protein 1 (VAP-1), dipeptidyl peptidase 4 (DPP-4), interleukin (IL)-33, low density lipoproteins (LDL), high density lipoproteins (HDL), reactive oxygen species (ROS), monocyte-adhesion molecule1 (MCP-1), matrix metallo-proteinases (MMP).*



Dysfunctional HDL in acute coronary syndrome patients

Our aim is to identify the individual contribution of HDL subclasses to HDL dysfunction (proteins, enzymes' activity and oxidative status) that make them dysfunctional, and estimate whether these modifications can discriminate between acute coronary syndrome (ACS) and stable angina (SA) CAD patients. To validate the data regarding the altered composition of HDL subclasses, we have evaluated in vitro their anti-inflammatory potential in TNF-α-activated human endothelial cells (EC) *(Carnuta M. et al., Sci. Rep. 2017).*

The new findings are:

- HDL_3 from CAD patients are more pro-oxidatively altered than HDL_2, expressed as increased content of oxidized-apoAI, ceruloplasm in and myeloperoxidase (MPO)/paraoxonase 1 (PON1) ratio. Consequently, HDL_3 from CAD patients become pro-inflammatory lipoproteins, despite of the intensive statin treatment of the patients.
- A panel of parameters such as apoCIII, MPO, oxidized-apoAI, ceruloplasmin, and PON1 correlates with the reduced anti-inflammatory potential of HDL_2 and HDL_3 from ACS compared to SA patients, and can discriminate between these groups of CAD patients.

2. IMPLICATION OF LIVER AND SMALL INTESTINE METABOLIC AXIS IN THE ATHEROSCLEROTIC PROCESS

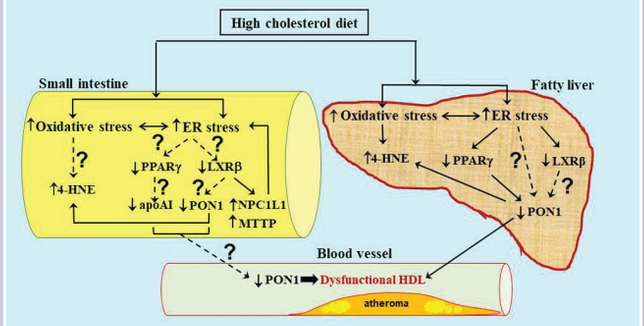
Dysfunctional HDL in hyperlipidemic hamsters

The goal of the study was to investigate the impact of a high fat diet on the mechanisms responsible for quality/functional HDL production by the small intestine and liver of hyperlipidemic hamsters *(Stancu C. et al., Molec. Nutr. Food Res. 2015).*

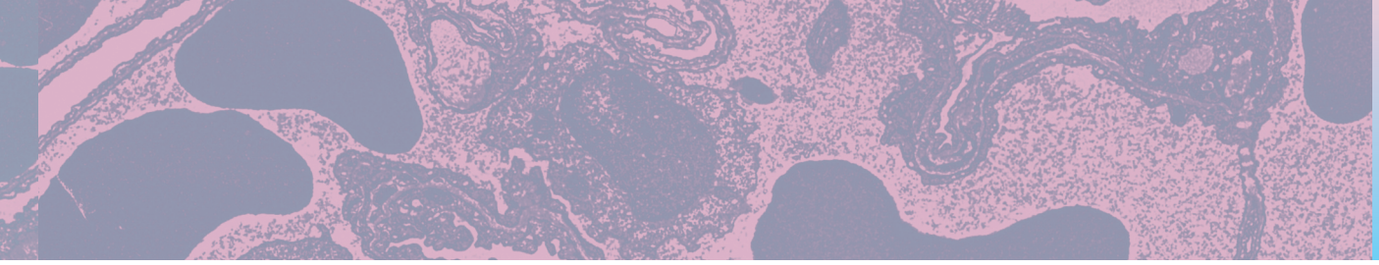
The new findings are:

The hyperlipidemic diet induces endoplasmic reticulum stress (ERS) and oxidative stress in the small intestine and diminishes protein synthesis of transcription regulators liver X receptors β (LXRβ) and peroxisome proliferator-activated receptor γ (PPAR γ).

Decreased LXRβ and PPARγ are associated with the reduction of apoAIand PON1 gene and protein expression, and increase of MPO levels, which favor the appearance of oxidized apoAI in HDL from hyperlipidemic hamsters and render HDL dysfunctional, that contributing to the development of lesion areas in the aortic valves.



Proposed mechanism for dysfunctional HDL production in the small intestine and liver under hyperlipidemic conditions. The high fat diet induces ERS in both organs and decrease the expression of LXRβ and PPARγ which are known to regulate the synthesis of HDL proteins (apoAI, PON1), MPO, ABC transporters, cholesterol transporter NPC1L1 and protein responsible for lipoprotein secretion.



3. EPIGENETIC FACTORS– MICRORNAS IN ATHERO-SCLEROSIS AND DIABETES

Biomarkers in hyperlipidemic and/or hyperglycemic patients

MicroRNAs (miRNAs) are small non-coding RNA sequences that regulate gene expression post-transcriptionally by translation inhibition or mRNA degradation. The molecular mechanisms involved in the cellular secretion of circulating miRNAs are not completely understood.We demonstrated their potential as risk biomarkers for cardiovascular and cardio-diabetic compli-cations. We have analyzed a panel of miRNAs from sera, modulated by hyper-lipidemia and/or hyperglycemia, and correlated them with biochemical parameters within lipid metabolism.*(Simionescu N., Niculescu L. et al., Molec. Biol. Rep. 2014)*.

The new findings are:

- MiR-125a-5p, miR-146a, miR-10a, miR-21 and miR-33a were increased in hyperlipidemic sera and correlated positively with sera's lipid and inflammatory parameters.
- Circulating miR-125a-5p and miR-146a levels were significantly increased in hyperglycemic and/or hyperlipidemic sera.
- A panel of circulating miRNAs was identifiedin hyperglycemic and/or hyperlipidemic patients'sera.
- A statistically significant correlation of the analyzed miRNAs with the increased lipids, CRP and IL-1β levels in hyperlipidemic and/or hyperglycemic sera, suggests a contribution of these miRNAs to the atherosclerotic process.

MiRNAs in HDLdiscriminate between stable angina and acute coronary syndrome patients

We identified a panel of specific serum miRNAs in circulating Lp, specifically in HDL, and demonstrated their association with vulnerable CAD patients *(Niculescu L. et al., Plos One 2015)*.

The new findings are:

- MiR-486, miR-92a, miR-122 havethe highest expression in sera from CAD patients relative to control following the cardiovascular disease-focused screening array.
- MiR-486, miR-92a, miR-122, miR-125a and miR-146a are associated mainly with serum HDL, and ten times less with IDL or LDL.
- Two circulating miRNAs, miR-486 and miR-92a, together with apoA-I and apoE, PON1 activity and the ratio HDL/LDL cholesterol can be used as an additional statistical tool to designate vulnerable CAD patients.
- A preferential distribution of miR-486 and miR-92a in the HDL subpopulations (HDL_2, HDL_3) can discriminate between vulnerable and stable CAD patients.

Hyperglycemia determines increased specific microRNAs levels in sera and HDL of acute coronary syndrome patients and stimulates microRNAs production in human macrophages

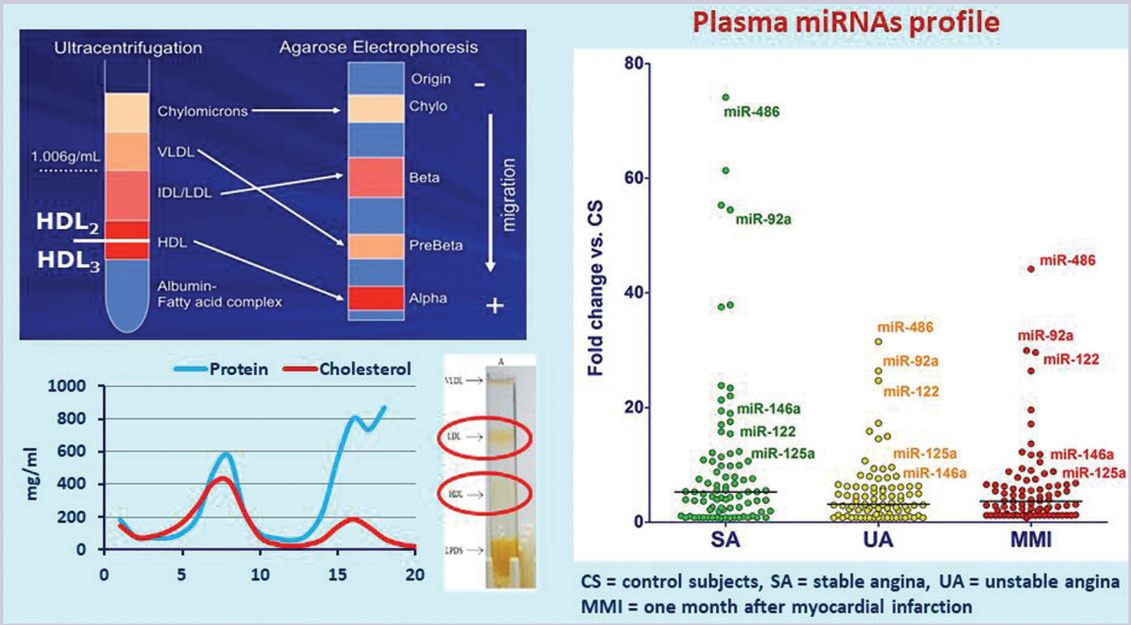
We have evaluated the levels of a panel of six miRNAs (miR-223, miR-92a, miR-486, miR-122, miR-125a and miR-146a) in sera and HDL subpopulations from stable angina (SA) and acute coronary syndrome (ACS) patients with or without hyperglycemia.

We have estimated the functional effects of ACS and SA patients' sera, with or without hyperglycemia, in cultured human macrophages, following the gene expression of the miRNAs processing machinery proteins (Dicer, Drosha, DGCR8) and cellular miRNAs production *(Simionescu N., Niculescu L. et al., Plos One 2016)*.

The new findings are:

- Increased levels of miR-223, miR-92a, miR-486 in seraand HDL subpopulations (HDL_2, HDL_3) correlate with hyperglycemia, and discriminate between ACS and SA patients.
- Exposure of human macrophages to ACS versus SA sera determines an increased production of miR-223, miR-92a, miR-486, miR-125a and miR-146a, by upregulation of Dicer, Drosha and DGCR8 expression, this effect being augmented by the increase of sera's glucose concentration.

In vivo modulation of miRNAs in hyperlipidemic hamsters

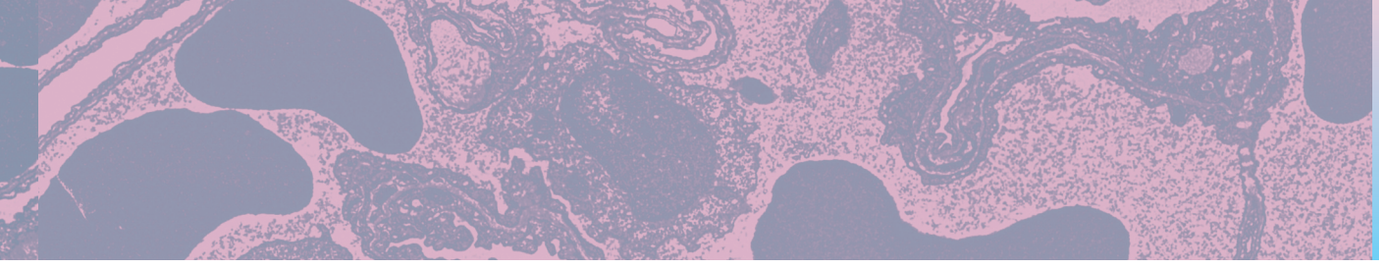


MicroRNAs screening array identified a panel of miRNAs in human plasma and in HDL subpopulations (HDL_2 and HDL_3), associated with hyperglycemia and/or hyperlipidemia, that can discriminate between vulnerable and stable angina CAD patients
(Niculescu L. et al., Plos One 2015).

Identifying those miRNAs specifically involved in dyslipidemia and cellular dysfunction is important to develop either a miRNA-based therapy or prognostic markers for the evolution of lipid-related diseases.We evaluated *in vivo* the potential of miR-486 and miR-92a inhibitors to reverse hyperlipidemia's effects in hyperlipidemic (HL) hamsters.We set up a combined approach involving bioinformatics analysis, 3'UTR cloning and *in vitro* experiments to identify and validate miR-486 and miR-92a target genes related to molecular mechanisms of lipid metabolism dysregulation *(Niculescu L. et al., Molec. Biol. Rep. 2018)*.

The new findings are:

- Plasma cholesterol levels and livers lipid content from HL hamsters were diminished as a result of the subcutaneously injected inhibitors specific for miR-486 and miR-92a.
- The restoration of hepatic SOAT2 and SREBF1 expression to normal levels after miR-486 inhibition, and of ABCG4, NPC1 and SOAT2 expression after miR-92a inhibition.



4. PHARMACOLOGIC ATTEMPTS TO ARREST OR REVERSE CARDIO-VASCULAR DISEASES BY MODULATING THE LIPID METABOLISM

Amlodipine effects in vivo (hyperlipidemic hamsters) and in vitro (human endothelial cells) (*Sima A. et al., J.Cell.Mol. Med. 2001; Toma L. et al., Biochem. Biophys. Res. Commun. 2011*).

***The new findings are**:*

• amlodipine exerts an anti-atherosclerotic protection in hyperlipidemic hamster by: (i) acting as an antioxidant agent, (ii) diminishing the uptake of LDL by the vessel wall, and consequently (iii) reducing the extent and size of the atherosclerotic lesions.

• amlodipine inhibits NADPH oxidase and ROS-sensitive inflammatory signaling molecules and induces positive effects on the endothelial-dependent mechanisms of NO biosynthesis by modulating eNOS and iNOS expression and their balance, thus decreasing cytotoxic peroxynitrites formation.

Statins pleiotropic effects in vivo (*Stancu C and A Sima, J. Cell.Molec. Med. 2001; Simionescu M., Stancu C. et al., Vasc. Pharmacol. 2002*).

***The new findings are**:*

• simvastatin administration to hyperlipidemic hamsters exerts an increase in plasma antioxidant potential, reduces transcytosis of Lp and restores the endothelial-dependent relaxation.

• simvastatin treatment determines an increase of the antioxidant potential in the sera from stable (SA) and unstable angina (UA) patients. Sera from simvastatin-treated SA or UA patients induce in macrophages and human aortic smooth muscle cells decreased accumulation of esterified cholesterol.

Caffeic acid reduces oxidative and inflammatory stress in hyperglycemic conditions in vitro (human endothelial cells) *(Toma L. et al., Biofactors 2017).*

***The new findings are**:*

• Caffeic acid (CAF) exerts anti-inflammatory effects in diabetic conditions: diminishes CRP, VCAM-1 and MCP-1 secretion by human endothelial cells exposed to glycated-LDL.

• Two new mechanisms of CAF's action were identified: (i) inhibition of RAGE expression, and (ii) attenuation of ERS in human endothelial cells.

Probiotics hypolipidemic effects and miRNAs modulation in hyperlipidemic hamsters *(Stancu C. et al., Molec. Nutr. Food Res. 2014; Niculescu L. et al., J. Funct. Foods 2019).*

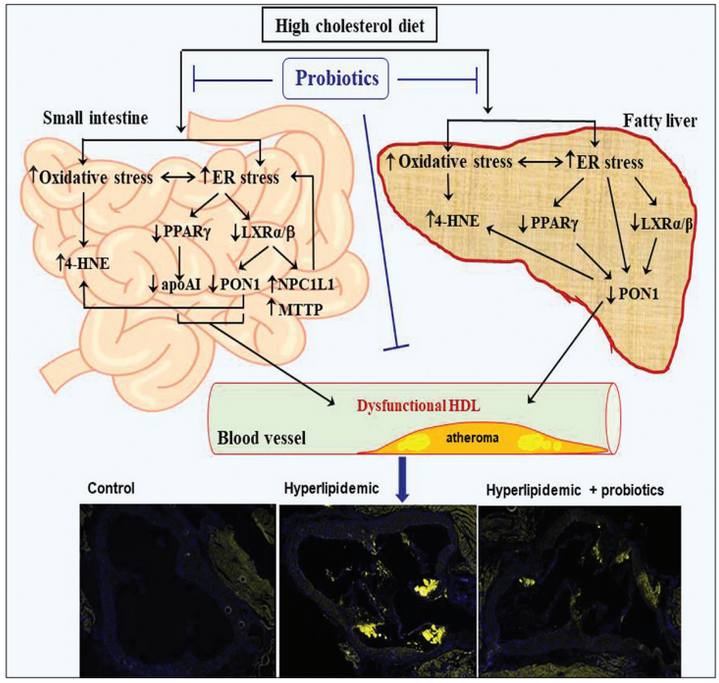
***The new findings are**:*

• probiotics exert: (i) lipid-lowering effect based on the complex entero-hepatic regulation of cholesterol metabolism; (ii) increased apolipoprotein and enzymes synthesis (apoA-I and PON1) mediated by upregulated nuclear receptors PPARγ/RXR and LXRs; (iii) protection against oxidative stress (decreased 4-HNE). The probiotic mix of *L. acidophilus* (LA-5) and *Bifidobacterium animalis subsp. Lactis (BB-12)* induces downregulation of Niemann Pick C1-like1 (NPC1L1) and Microsomal Triglyceride Transfer Protein (MTTP) in the small intestine.

We investigated the possible connections between dietary interventions and the RNA-related epigenetic processes by analyzing the *in vivo* effects of probiotics treatment or high-fat diet (HFD) withdrawal on serum and hepatic levels of a selected panel of lipid-related miRNAs (miR-223, miR-122, miR-486, miR-92a) in HL hamsters *(Niculescu L. et al., J. Funct. Foods 2019)*.

The new findings are:

- Probiotics administration or the HFD arrest reduce serum and hepatic lipid levels, leading to a significant lipid clearance of the steatotic liver and decrease of the serum and hepatic expression of miR-223, miR-122a, miR-92a and miR-486.



- The hypolipidemic dietary interventions affect differently the genetic (cholesterol metabolism-related genes: LDLR, HMGCS1, HMGCR, SR-BI) and the RNA-related epigenetic factors (Dicer, DGCR8) in the HL livers.

Ginger extract diminishes oxidative and inflammatory stress in vitro (human endothelial cells) and in vivo (hyperlipidemic hamsters)
(Carnuta M. et al., Phytomedicine 2018; Toma L. et al., J. Funct. Foods 2018; Stancu C. et al., Molec. Nutr. Food Res. 2019).

The new findings are:

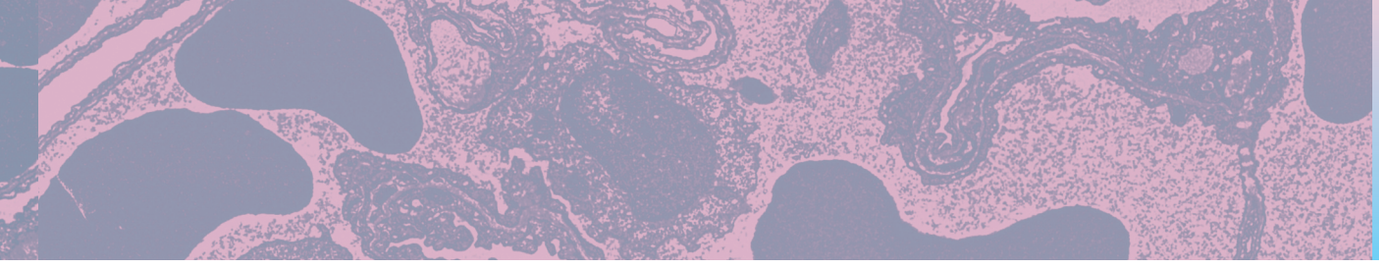
- Ginger extract (GEx) exerts anti-inflammatory action by decreasing ninjurin 1 (Ninj-1), TNFα receptor 1 and NADPH oxidase subunits expression and increasing sRAGE levels in the culture media of TNFα-exposed human endothelial cells.
- In hamsters livers, the simultaneous administration of GEx with the high-fat diet induced the diminution of steroyl-CoA desaturase (SCD1) gene expression and estimated activity. GEx induced the inhibition of ERS and the increase of LXRα/β and PPARγ protein expression, leading to increased synthesis of ABCG5/G8 and CYP7A1 proteins and the decrease of accumulated cholesterol.
- In hamsters plasma and small intestine, GEx reduces the oxidized-apoAI levels, induced by the high-fat diet, by diminishing the MPO/PON1 ratio; these effects are associated with the retrieval of SIRT1-LXRα/β-PPARγ pathway, that further adjust the levels of MPO, PON1 and ABC transporters.
- These results may represent a substantial support to develop a new strategy for lowering the lipid accumulation and oxidative stress in the steatotic liver and small intestine.

Database repository for miRNAs profiling

MicroRNA expression profiles in different tissues from normolipidemic hamster and high-fat diet-induced hyperlipidemic hamster (*Mesocricetus auratus*), *Niculescu L. et al., GEO database GSE128226*, publication date 13 March 2019.

PERSPECTIVES

The understanding of the molecular mechanisms revealed by our projects will help decipher the complexity of gene regulation and signaling mechanisms modulated by atherogenic lipids, highlight new biomarkers for cardiovascular diseases and identify new therapeutic molecules to improve the life of many people affected by atherosclerosis, diabetes, metabolic syndrome and obesity.



COLLABORATION

INTERNATIONAL

•**2003-2007** *APOA5 mechanisms in triglyceride metabolism* - **Prof. Jean-Charles Fruchart** and **Dr. Jamila Fruchart-Najib**, Institute Pasteur of Lille and University of Lille 2, Lille, France (*Fruchart-Najib J. et al., Biochem.Biophys. Res.Commun. 2004; Niculescu L. et al., Clin Chem Lab. Med. 2007*).

• **2004-2005** *The control of lipid metabolizing enzymes in hyperlipidemic hamsters*- **Prof. Kyriakos Kypreos**, Pharmacology Laboratory, University of Patras Medical School, Patras, Greece.

• **2006** *Determination of IRS1 and IGF1R gene polymorphisms in patients with cardiovascular diseases by using gene array and qPCR techniques* - **Prof. Danilo Norata**, Department for Pharmacological Sciences, Milano University, Milan, Italy.

• **2006** *Effects of glycated LDL on scavenger receptors from human smooth muscle cells*- **Prof. Lina Badimon**, Cardiovascular Research Center CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain.

• **2008** *Methods to evaluate the serum antioxidant potential- paraoxonase1 (PON1) activity* - **Dr**. **Hagai Tavori**, Laboratory of Natural Medicinal Compounds, MIGAL Galilee Technology Center, Haifa, Israel.

• **2008-2011** *HDL associated enzymes in cardiovascular disease* - **Prof. Matti Jauhiainen** and **Dr. Marius R. Robciuc**, National Institute for Health and Welfare, Helsinki, Finland (*Robciuc M. et al., Lipids Health Dis. 2010; Niculescu L., et al., Biochem. Biophys. Res. Commun. 2011*).

• **2012** *Immunodetection of molecules specific for lipid metabolism, in tissues or cultured cells, by confocal microscopy* - **Prof. Jorg Hereen**, Department of Biochemistry and Molecular Cell Biology, University Medical Clinic Hamburg-Eppendorf, Hamburg, Germany.

• **2012** *MiRNAs analysis techniques in biological samples* - **Prof. Christian Weber,** Institute for Cardiovascular Prevention (IPEK), Ludwig-Maximilians Universität, Munich, Germany.

• **2012-2015** *Molecular interactions of oxidized lipoproteins with vascular endothelial cells* - **Prof. Shlomo Sasson**, Hebrew University, Jerusalem, Israel (*Stancu C. et al., Molec. Nutr. Food Res. 2015; Riahi Y., ..., Simionescu M., Sima A., ...,Sasson S., J. Cell. Mol. Med. 2015*).

• **2014** *Functional analysis and bioinformatics of microRNAs* - **Prof. Leon de Windt**, Molecular Cardiology department, Cardiovascular Research Institute (CARIM), Maastricht University, Maastricht, The Netherlands (*Niculescu L. et al., Molec. Biol. Rep. 2018*).

• **2014-2019** *Bioinformatic analysis of microarray microRNAs distribution in hyperlipidemic hamster tissues* - **Dr. Yvan Devaux**, Cardiovascular Research Unit, Luxembourg Institute of Health, Luxembourg (*Niculescu L. et al., GEO database GSE128226, 2019* and COST Action CA17129 *"Catalysing transcriptomics research in cardiovascular disease"* 2018-2022, CardioRNA network, https://cardiorna.eu/).

NATIONAL

• **2003-2005** Romanian Academy "F. Rainer" Institute of Antropology **(Dr. Cristina Glavce)**

• **2003-2010** National Institute for Diabetes, Nutrition and Metabolic Diseases „N. Paulescu" (**Prof. Dr. Constantin Ionescu Târgoviște**, **Dr. Maria Vlădică**);

• **2006-2008** University of Medicine and Pharmacy „Carol Davila" and Cardiology Clinic of University Emergency Hospital Floreasca (**Prof. Dr. Maria Dorobanţu**);

• **2006-2008** National Endocrinology Institute „C.I. Parhon" (**Dr. Olga Ianăş**);

• **2007-2010** University of Medicine and Pharmacy „Carol Davila" (**Prof. Dr. Denisa Margină**)

• **2006-2010** National Institute for Food Chemistry **(Dr. Floarea Serbancea)**;

• **2012-2016** University of Medicine and Pharmacy „Carol Davila" and Cardiology Clinic of University Emergency Hospital Elias (**Prof. Dr. Doina R. Dimulescu**);

• **2016-2019** Cardiovascular Clinic of the "Agrippa Ionescu" Emergency Hospital **(Dr. Viorel Goleanu, Dr. Oriana Moraru**).

INTERNATIONAL GRANTS

• **2001-2004** Grant FP5 ICA1-CT-2000-70020, Centre of Excellence of the European Community, *Function and dysfunction of blood vessels: transcytosis in normal and pathological states, alterations in atherosclerosis and diabetes; their therapeutic control*.

• **2002-2004** NATO SCIENCE PROGRAMME, *Role of ApoE in Cholesterol and Triglycerides Homeostasis.*

• **2005-2007** Grant FP6, SSA-EC 16873 *Strengthening the European Research Area by Reinforcement of Romanian Research Competency in Genomics and Proteomics of Major Global Risk Diseases: Atherosclerosis, Diabetes and its Complications*.

• **2008-2011** COST Action BM0602 (WG3, WG 4) *Adipose Tissue: A Key Target for Prevention of the Metabolic Syndrome*, European Community Funds.

• **2008-2012** POS-CCE 143/SMIS CSNR 2667 *Extension and modernization of the research infrastructure in order to increase competitiveness in the field of cardiovascular diseases, diabetes and obesity (CARDIPPRO)*, European Community Funds.

• **2014-2015** POS-CCE ID-1877/SMIS-CSNR 49154 *Structuration of a new compartment for cellular sorting and tissue cryo-preservation for research and therapeutic purposes (SORTIS)*, European Community Funds.

• **2018-2022** COST Action CA17129 "*Catalyzing transcriptomics research in cardiovascular disease*" (CardioRNA network), European Community Funds.

GRANTS AWARDED BY COMPETITION (2001- 2019)

Studies in patients with cardiovascular diseases and/or diabetes.

Cellular and molecular mechanisms of dyslipidemia

• **2003-2005 VIASAN PNCDI Grant**, partners: National Institute for Diabetes, Nutrition and Metabolic Diseases „N. Paulescu" and Anthropology Institute „Fr. Rainer", *The impact of obesity in generating diabetes and cardiovascular diseases in urban communities from Romania - a population, physio-pathologic and genetic study* (OBEDIAGEN).

• **2007-2010 PARTNERSHIP PNCDI-2 Grant**, partners: University of Medicine and Pharmacy „Carol Davila", National Institute for Food Chemistry and National Institute for Diabetes, Nutrition and Metabolic Diseases „N. Paulescu", *The study of the cellular, molecular and genetic mechanisms by which dyslipidemia induces insulin resistance; identification of probiotic active compounds and treatment methods* (LIPIDERI).

• **2012-2016 PARTNERSHIP PNCDI-2 Grant**, partner: Cardiology Clinic of University Emergency Hospital Elias, *New predictive biomarkers for the evolution of the stable and unstable coronary artery disease identified by lipidomic, proteomic and molecular biology technologies* (BIOMARCAD).

Genetics, epigenetics and molecular biology

• **2004-2006 BIOTECH PNCDI Grant**, *The employment of APOA5 and APOE gene polymorphisms as molecular markers in the*

study of evaluation of genetic risk factors of subjects with obesity and its associated disorders (diabetes, hypertension and atherosclerosis).

• **2008-2011 IDEI PNCDI-2 Grant**, *Molecular strategies for the reversal of atherosclerotic process by the modulation of secretion and cellular signaling pathways and intracellular assembly of anti-atherogenic lipoprotein*s.

• **2015-2017 Young Teams Research Grant**, *Assessment of molecular strategies to improve atherogenic dyslipidemia by modulating the microRNAs expression* (THERAMIR).

Studies in experimental models of the molecular mechanism of lipoprotein metabolism

• **2001-2003 VIASAN PNCDI Grant**, *The role of endothelium in atheroma formation: the comparative study on protective effect of various statins on cells from atherosclerotic plaque*.

• **2005-2007 IDEI PNCDI Grant**, *The role of transcription factors PPARa and PPARg in the regulation of genes for atherogenic lipoprotein receptors on endothelial and smooth muscle cells*.

• **2015-2017 Young Teams Research Grant**, *Molecular mechanisms of hyperlipidemia-induced insulin resistance; metabolic connections between the intestine, liver steatosis and atherosclerosis* (MECLIPINSMETAB).

AWARDS

Romanian Academy "Victor Babeș" Prize, for experimental studies of cellular and molecular events initiating atherosclerosis, **1990** (A. V. Sima, R. Mora).

"Constantin Velican" Romanian Society for Cell Biology Prize: **1994** (A.V. Sima), **1995** (A.Dobrian), **1998** (D. Tirziu), **2010** (C.S. Stancu), **2017** (L. S. Niculescu).

"**Sanofi" Thrombosis Award** - for Atherosclerosis and Thrombosis Research, for clinical and laboratory research on atherothrombosis, **1998** (A. V. Sima, C. S. Stancu),

"Maya and Nicolae Simionescu" Award of the Romanian Society for Cell Biology for research on cellular and molecular biology and pathology, **1999** (A. V. Sima).

"Images in Cellular – Molecular Medicine" Award of the Foundation of Cellular and Molecular Medicine and the Journal of Cellular and Molecular Medicine, **2003** (A. V. Sima).

„Herbert Berler" Award for Excellence in Research, **2012** (L. S. Niculescu, G. M. Sanda, A. V. Sima).

Romanian Academy "Nicolae Simionescu" Prize, **2015** (C. Stancu, L. S. Niculescu).

Romanian Cardiology Society Award for Excellence in Research, **2016** (The Lipidomics team).

The Lipidomics Team and
Dr. Maya Simionescu
at the 84th European Atherosclerosis Society
(EAS) Congress, Innsbruck, Austria,
29 May - 1 June 2016.