DEPARTMENT OF BIOPATHOLOGY AND THERAPY OF INFLAMMATION

Elena Butoi, *Ph.D.*

Head of Laboratory
E-mail:
elena.dragomir@icbp.ro

INFLAMMATION RESEARCH LABORATORY

Major position/appointments

- Principal Investigator, Scientific Researcher grade I
- Supervision of Graduate Students and Postdoctoral Fellows
- Expert evaluator of the national grants
- Invited Peer Reviewer for International Scientific Journals

STAFF

Monica Țucureanu, *PhD* / ***Ana Maria Gan,*** *PhD* /
Sergiu Cecoltan, *PhD student* / ***Mihaela Vădana,*** *PhD student* /
Răzvan Macarie, *PhD student* / ***Letiția Ciortan,*** *PhD student* /
Andreea Mihăilă, *Master student* / ***Gabriela Meșca,*** *Technical assistant*

SELECTED NEW FINDINGS OF THE LABORATORY

MOLECULAR AND CELLULAR MECHANISMS OF CARDIOVASCULAR DISORDERS

• Enoxaparin reduces monocyte adhesion to TNF-α, LPS-, or high glucose-activated EC

• Aspirin corrects the high glucose-induced changes in intracellular calcium homeostasis and NO production in human EC

• PPARα activators (fenofibrate and clofibrate) inhibit MCP-1 and fractalkine expression induced by high glucose in human smooth muscle cells

• Identification of early structural and functional changes induced by diabetes in the aortic valve in vivo and establishing significant correlations between inflammatory, remodeling and calcification markers and functional and serum parameters

• Development of a 3D construct of human aortic valve as a model to study calcification mechanisms in aortic valve disease

• In diabetic conditions, upon cross-talk with macrophages, smooth muscle cells exhibit augmented expression of MMP-1 and MMP-9 and higher levels of soluble MCP-1 which is functionally active and involved in MMPs regulation

• Cross-talk between macrophages and smooth muscle cells amplifies inflammation in macrophages, impairs collagen and metalloprotease synthesis and promote angiogenesis

• Functional analysis of the fractalkine gene promoter in human aortic smooth muscle cells exposed to proinflammatory conditions

• Subendothelial resistin enhances monocyte transmigration in a co-culture of human endothelial and smooth muscle cells by mechanisms involving fractalkine, MCP-1 and activation of TLR4 and Gi/o proteins signaling

• Monocytes and smooth muscle cells cross-talk activates STAT3 and induces resistin and reactive oxygen species production.

• Resistin has pro-inflammatory effects on human smooth muscle cells: up-regulates fractalkine and its receptor, CX3CR1 expression by TLR4 and Gi-protein pathways.

• A novel pro-inflammatory mechanism of action of resistin in human endothelial cells: up-regulation of SOCS3 expression through STAT3 activation.

INFLAMMATION RESEARCH LABORATORY

CURRENT PROJECTS

1. TARGETED THERAPIES FOR DIABETES - RELATED AORTIC VALVE DISEASE – THERAVALDIS, A MULTI-LABORATORIES PROJECT

Aortic valve disease and especially calcific aortic valve disease (CAVD) is a global health burden in all aging societies, including the Romanian population. It is known that the presence of diabetes accelerates CAVD, and is predictive of poor prognosis in valve disease and of faster degeneration of implanted bio-prosthetic aortic valves. To our knowledge, a clinically viable pharmacological therapy for valve disease is still not available, the only alternative being the invasive and costly valve replacement. This urges the need for additional research to identify distinctive mechanisms of valve disease progression.

THERAVALDIS' objective is to advance the understanding of the mechanisms of aortic valve disease in the diabetic milieu in order to discover and validate new possible targets for nanotherapy and stem cell therapy;

Hypothesis: that upon the identification of distinct diabetes-related changes in valvular cells and matrix components from relevant in vivo models, we could target the major alterations with appropriate therapy as treatment for valve diseases. The three **main pathogenic processes** envisioned as putative targets are: endothelial to mesenchymal transition (EndMT), ectopic osteogenesis in the valves and dysfunctional recruitment and homing of progenitor cells.

Ongoing experiments are focused on:

• Characterization of the early and progressive changes induced by diabetes in the aortic valves in vivo, as well as in the circulating endothelial progenitor cells (EPC). This will advance the understanding of aortic valve disease and will indicate new possible biomarkers and new targets for therapies.

• Developing of in vitro 3D models of aortic valve leaflet seeded internally with human valvular interstitial cells (VICs) and externally with human valvular endothelial cells (VECs) to validate the specific terapeutic targets revealed by our results.

• Tests and validation of new therapeutic strategies: (a) *targeted nanotherapeutics*;(b) *stem cell therapy*

• Preclinical validation of the most efficient nano and/or stem cell-based therapies *in vivo*.

Methods employed and Results obtained:

We evaluated changes of aortic valve function and changes associated with inflammation, ECM-remodelling, and calcification induced in a hyperlipemic ApoE-/- mouse model by early type I diabetes onset (at 4 and 7 days after streptozotocin induction).

The hemodynamic valve parameters were evaluated by echography and blood samples and aortic valves were collected.

Plasma parameters were measured and inflammatory, remodelling and osteogenic markers were evaluated in the aortic valves. Correlations between all parameters were determined.

MAIN FINDINGS :

• **Diabetes induces early functional alterations in the aortic valves of hyperlipemic ApoE-/- mice.** The results revealed that the mean value of velocity time integrals (VTI) was significantly increased both for D4 group compared to C4 group (by ~1.56 times,) and for D7 group compared to C7 group (by ~1.77 times) and the mean value of transvalvular velocity (VEL) was significantly higher for D7 group vs C7 group (by ~ 1.80 times).

• **The aortic valves of hyperlipemic ApoE-/- diabetic mice exhibit increased expression of inflammatory markers:P-selectin, ICAM-1, VCAM-1, PECAM-1 expression, as well as TGFβ family members: TGFβ1, BMP2, and BMP4.**

• The aortic valve of hyperlipemic ApoE-/- diabetic mice display enhanced expression of pro-osteogenic markers: alkaline phosphatase (ALPL), osteopontin and osteocalcin, cell activation markers, fibronectin and remodeling molecules: MMP2 and MMP9

• Correlations between aortic valve tissue markers, plasma parameters and hemodynamic parameters in hyperlipemic ApoE-/- diabetic mice were established

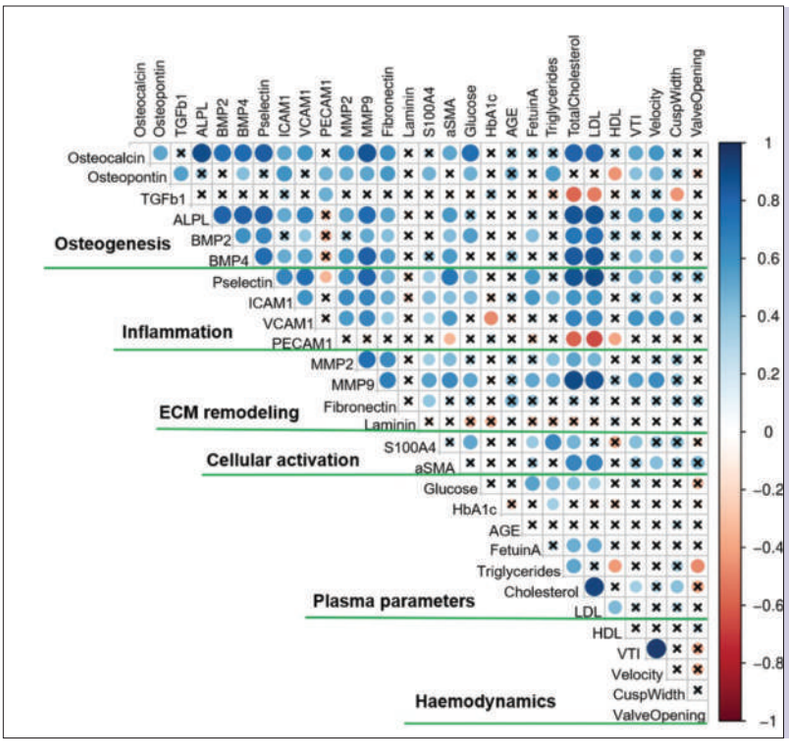
To gain insight into the possible associations between hemodynamic parameters, tissue markers and plasma parameters determined in this experimental setting, we performed a correlation analysis.Our analysis revealed that:

• peak aortic jet velocity was highly correlated with inflammatory biomarker VCAM-1, pro-osteogenic markers osteocalcin and ALPL, remodeling enzyme MMP9 and myofibroblast marker αSMA;

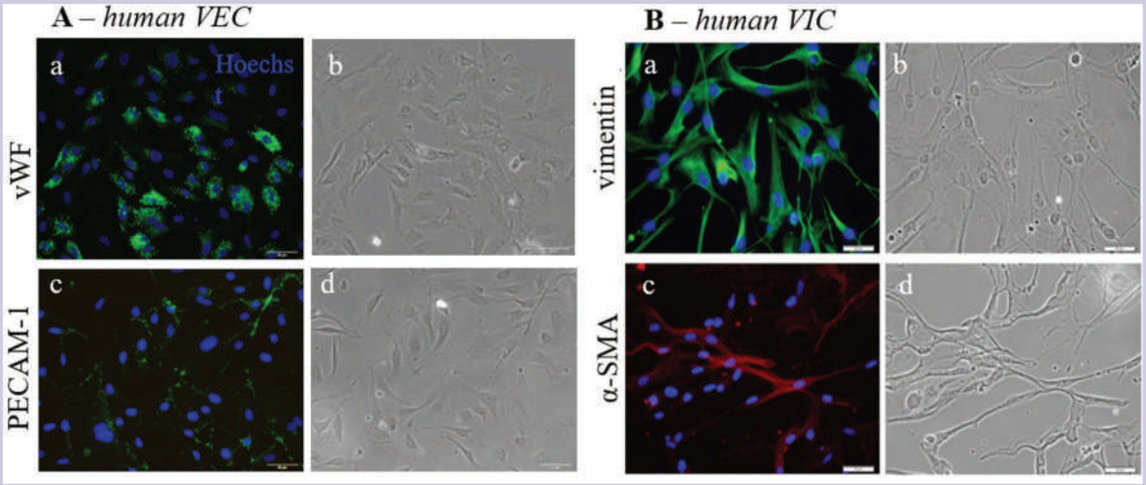
• glycemia was significantly correlated with: osteocalcin, osteopontin, BMP2, P-selectin, ICAM-1, MMP9, S100A4, Fetuin A, triglycerides, total cholesterol and LDL-cholesterol.

• very high correlations ($r > 0.8$) were found between: P-selectin and plasma parameters: LDL and total cholesterol, osteoblastic markers osteocalcin and ALPL and remodeling enzyme MMP9; BMP2 and ALPL, BMP4 and total cholesterol, LDL, ALPL and MMP9; MMP9 with total cholesterol, LDL, P-selectin; ALPL with total cholesterol, LDL, P-selectin, BMP4, osteocalcin;

• high correlations ($0.5<r<0.8$) were found between: P-selectin with Fetuin A, VCAM-1, ICAM-1, BMP2, BMP4, MMP2, α-SMA, VTI; VCAM-1 with total cholesterol, LDL, P-selectin, ICAM-1, BMP4, osteocalcin, ALPL, MMP9, MMP2, αSMA and the hemodynamic parameters VTI and velocity. ICAM-1 displayed a positive high correlation with total cholesterol, LDL, Fetuin A, P-selectin, VCAM-1, osteopontin, osteocalcin, with ALPL, MMP9, MMP-2 and with fibronectin. PECAM-1 displayed a negative high correlation with the seric parameters total cholesterol and LDL.



Correlation matrix between pro-osteogenic, inflammatory, ECM remodelling, cellular activation, plasmatic and hemodynamic parameters. *Mean values of measured parameters for each experimental group were used for a Pearson correlation matrix conducted using the R package "corrplot". Blue represents positive correlation; red represents negative correlation; darker colours.*

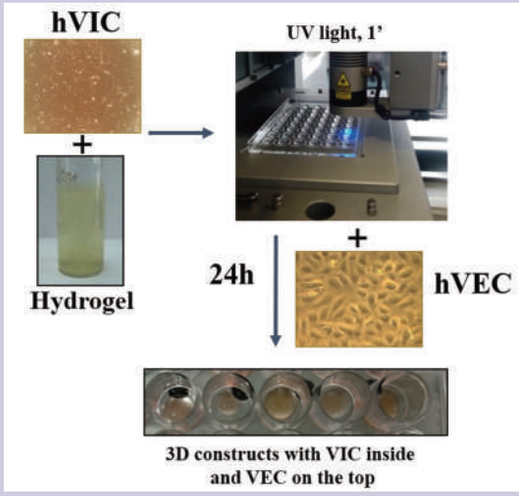


Expression of valve endothelial (A) and interstitial (B) cells specific markers in 2D culture; obtaining of the 3D-construct. ***A.*** *Positive fluorescent staining for (a) von Willebrand Factor and (c) CD31(PECAM-1) in the human aortic valve endothelial cells isolated from human operated valve. b, d– contrast phase images.* ***B.*** *Positive fluorescent staining for (a) vimentin and (c) alpha-smooth muscle actin (α-SMA) of human aortic valve interstitial cell. DAPI was used as a counterstain; scale bar=50 μm.*

Development of an *in vitro* 3D model of human aortic valve leaflet.

To develop 3D in vitro models**,** initially, we isolated and characterized the human valvular endothelial cells (VECs) and valvular interstitial cells (VICs) from human aortic operated valves. Aortic VECs are positive for endothelial markers von Willebrand factor (vWF) and PECAM-1 and VICs are positive for α-SMA and vimentin protein.

After cells isolation and characterization, human VEC and VIC were used for development of a 3D construct similar with human valve leaflet for study of calcific valve disease. Thus, VICs were encapsulated in a methacrylated gelatin-based hydrogel (GP-MA) followed by exposure for 1 minute to UV light for crosslinking. Subsequently VECs were cultivated on topand the 3D constructs were placed in DMEM and cultured for different period of time.

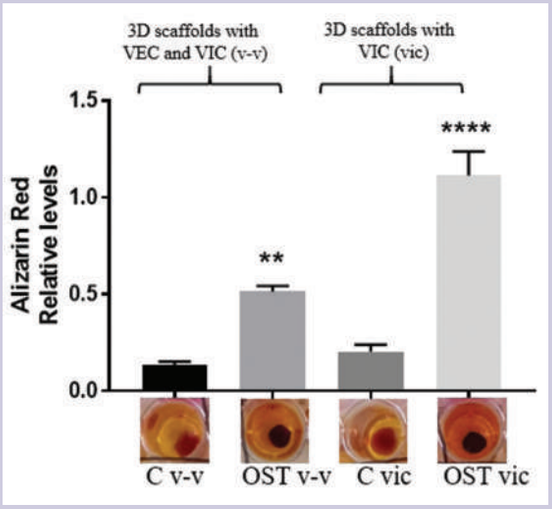


3D-construct

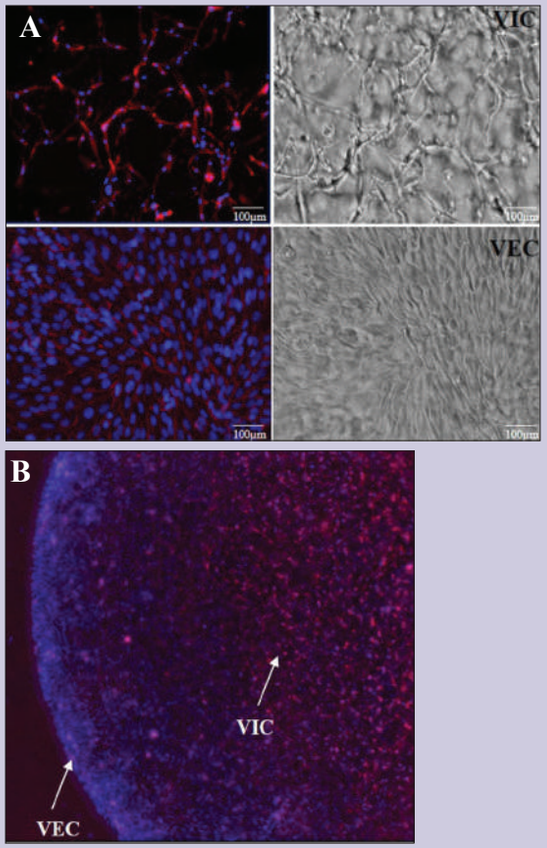
Human VICs were re-suspended in GP-MA hydrogel solution (gelatin methacrylate (10%), 1% alginate and Photo-initiator - Igracure 2595). 100 μL of the VICs-laden prepolymer solution was drop-wise added on a sterilized glass side covered with a 48-hole (Ø - 8 mm, 1 mm thickness) and subsequently crosslinked by exposure for 1 minute to UV light (365nm) using 3DDiscovery® bioprinter (RegenHu). Cell-laden hydrogels were removed from the glass slide and cultured, according to protocol. After 24h, VECs (5x10^5/cm^2) were added on the top of 3D constructs.

Started with the second day, encapsulated VICs in 3D constructs started to gain a fibroblast-like phenotype with elongated-shape appearance, and were homogeneously distributed through the entire hydrogel thickness. VECs cultured on top of the 3D construct grow, proliferate and form a monolayer over the hydrogel, as show in the phase contrast picture or TRITC-phalloidin and Hoechst staining of construct.

Functionality of our 3D model to study the mechanisms of calcific valve diseases was tested by exposure of 3D constructs with human VICs or with VEC-VIC to osteogenic stimuli for 14 days. Alizarin Red experiments revealed the calcific nodule formation (increased calcium staining) in 3D constructs exposed to osteogenic media compared with constructs grown in normal culture media.



Osteogenic environment activates VICs in 3D constructs. A. *Alizarin red was used to stain the mineralized nodules formed by cells from 3D constructs with VIC or 3D constructs with VEC and VIC (v-v) cultured for 14 days. The lower panel showed the representative stained construct from each of the experimental group. The upper graph displayed the quantitative measurement of Alizarin red dye released from the mineralized nodules formed in 3D constructs cultured in normal conditions or exposed to osteogenic media (OST). n = 3, **p < 0.001, ****p < 0.0001.*



Morphology of VICs and VECs from 3D constructs.

A*. The cell morphology in 3D construct at day 7 of culture as determined by phalloidin labelled F-actin (red) and DAPI nuclear staining (blue) – left side. Hydrogel formula supports cell network development of VICs (inside the hydrogel) and VECs proliferation as a monolayer on the scaffold surface. Contrast phase images of the surface and inside of hydrogel with VICs encapsulated inside and VECs cultured on top - right side. Scale bar indicates 100 µm.*

B: *3D reconstruction of multiple images of a 3D construct, realized by z-stack option of cellSense software – median image.*

Ongoing experiments investigate molecular mechanism of valvular calcification in 3D construct exposed to diabetic conditions (high glucose concentration).