PROGRESS REPORT – 2nd year

Runtime of the project:
x☐ 2nd year

Period covered:
From 12 /06/2018 to 12/05/2019

Acronym	XploreCAD
Project Title	Advanced ex vivo analyses and multi-frequency ultrasound technology for improved evaluation and diagnosis of coronary plaque
Start Date Consortium	December 19th, 2017
Date of the Report	March 2019

PARTNERS

Number	Principal Investigator	Organisation	Country
1 (Coordinator)	Rune HANSEN	Stiftelsen SINTEF, Trondheim	Noeway
2	Felicia ANTOHE	Institute of Cellular Biology and Pathology N. Simionescu, Bucharest	Romania
3	Mathieu LEGROS	Vermon, SA, Tours	France
4 (Associated partner)	Kenneth Caidahl	Karolinska University Hospital	Sweden

1. Achievement of planned objectives

Describe the activities that have been performed during the year to meet the objectives given in the proposal. Outline the work carried out by each partner. Explain the progress of the work in line with the work plan (including milestones and deliverables). Estimate the current degree of completion of the objectives (Max. 2 pages).

The activities performed in this period were fully complete according to the Gantt diagram. The M1.1a was completed and the M1.2a was started with bioinformatics analysis and validation of the results generated by mass spectrometry analysis. Simultaneously selection and significance of the data will be performed to finish the proposed milestones and to build the reports for M1.2a; M1.1b; M2.1 in due time (end of 2020).

***In vivo* experimental design and preliminary results**

As mentioned in the first year report the experimental lot of 40 rabbits described in the project was split in two because of the limited logistical capacity of the subcontracted animal facility located at National Institute of Research and Development Cantacuzino (INCDMM), Contract No. A420, 23/04/2018. Thus the first lot of 20 rabbits (Stage I) was developed and completed between: August and November 2018. The next lot (Stage II) was developed between March and June 2019. At this time all the animals were processed and tissue samples and blood were harvested and deposited at low temperature to be analysed. The partial preliminary results of the Stage I (M1.1a) were reported on December 5th, 2018 to the UEFISCDI, the financial authority and could be found at the following web

site address: http://www.icbp.ro/static/en/en-networking_grants-grants-international/xplorecad.html.

The WP2 of the full proposal was focused on the evaluation of selected alarmins in plaques and sera of atherosclerotic patients. Up until this moment, patient enrollment and sample collection have been performed, as initially proposed, by KUH partner.

Related to the alternatives described in the WP2 tasks about the proteomic evaluation of plaques/serum from atherosclerotic patients, we all decided according to the suggestion of Ulf Hedin to skip the part related to proteomics on human plaques because this to a large extent already was done within their BIKE project.

Since part of the activities, concerning the mass spectrometric analyses have already been performed by KUH partner during another study, the ICBP partner remains with the on-going tasks of confronting and corroborating the results coming from the aortic, carotid and coronary plaque mass spectrometric analyses from the atherosclerotic rabbit model with the results obtained by the KUH partner from human carotid plaques. Qualitative and semi-quantitative data from the two types of samples will be compared using Protein Center LIMS bioinformatics platform for proper data management and relevant projection of alarmin biomarker pattern potential in order to propose selected molecules for microbubble targeting purposes and detection using the proposed ultrasound transducer.

By analyzing blood and plaque samples from both patients and animal models, the XploreCAD project will identify potential biomarkers of coronary artery disease. Using a new atherosclerotic animal model, the New Zeeland White Rabbits exposed to hyperlipidemia in the presence or absence of statins in association with PCSK9 inhibitors, we can safely assume that the high-performance technologies proposed will evidence modified profiles of pro- inflammatory cytokines, adhesion factors and regulatory proteins that can reduce atheroma plaques for the benefit of human health. In Figure 1 the general protocol followed to implement the project is shown as an interconnected diagram.

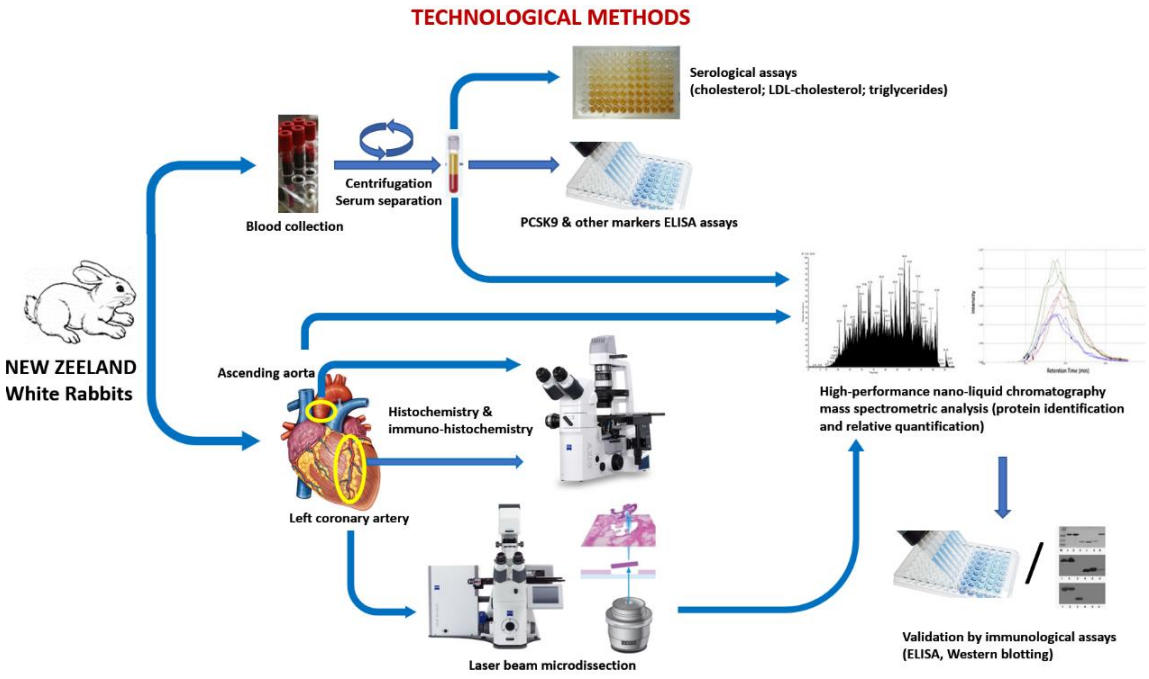

***Figure 1**. The novel design of the experimental model for atherosclerosis and the smart selection of clinical, biochemical and mass spectrometry tecnologies were carrefuly selected to unreveal the potenial biomarkers responsible for the residual risks in cardiovascular disease under agresive treatment with low lipid drugs.*

Progress / Intermediary results

After 12 weeks the tissue fragments harvested from all animal groups demonstrated that atherosclerotic advanced unstable plaques were developed in the lesion pron-areas of all animals fed high fat diet and the applied treatment reduced significantly the plaques dimension and also the histologycal aspects of the lesion.
Significant macroscopic modification could be observed when the animals were euthanized and the aorta, heart, carotid arteries tissue fragments were harvested from each animal. In Fig. 2, upper pannel, the general aspect of the liver can be visualised and is indicative of the effects of the hyperlipidemic diet. The lower pannel of Fig. 2 demonstrated severe lipid deposits in a representative Oil Red O coronary artery stained section, as opposed to the C and As animals.

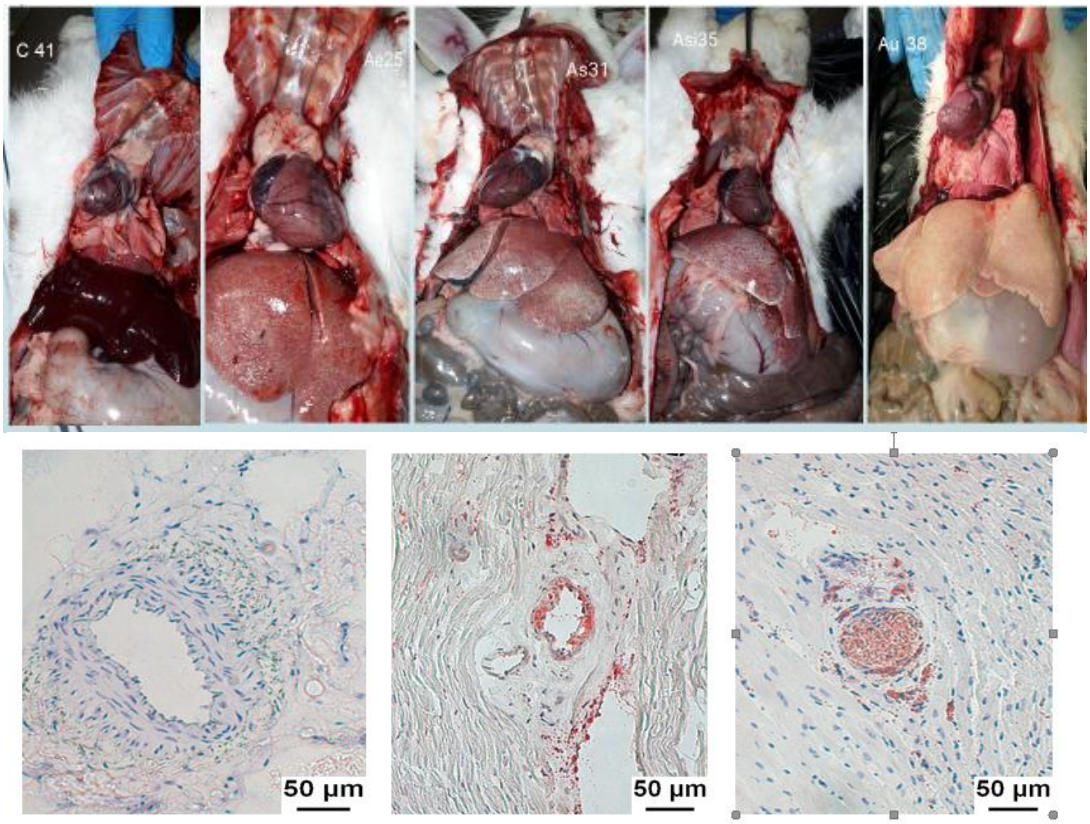

Figure 2 *.The macroscopic aspects of the internal organs of C, Ae, As, Asi and Au rabits (from left to right) at the end of the experiment (after 12 weeks) are presented in the upper pannel. Representative hematoxilin and lipid specific Oil Red O dye stainings of coronary artery frozen sections of C, As and Au (from left to right) animals are depicted in the lower pannel.*

One of the highlights of the XploreCAD project was the analysis of molecules or molecular patterns such as alarmins, to discriminate the different stages of atheroma plaque progression and better explain the residual risk of cardiovascular-related events and mortality after treatment with conventional and/or modern specific drugs. These molecules are a class of multifunctional endogenous proteins released under long-lasting stress factors, such as the hyperlipidemic diet.
In this regard, the ICBP partner has been responsible for the *in vivo* experimental design in order to assess and reveal specific patterns of alarmins and other molecules in early/ vulnerable plaques of hyperlipidemic animals when compared to appropriate controls. The proposed mass spectrometry-based proteomic analysis tackles both the local, tissue level of alarmins (and other significantly altered resident molecules) but also their circulating levels inside the blood flow.
The hyperlipidemic, as well as the animal groups which received treatments were evaluated at different time points for plasma lipid and glucose levels using specific assay kits from DIALAB GMBH (Wiener Neudorf, Austria) (Fig. 3).

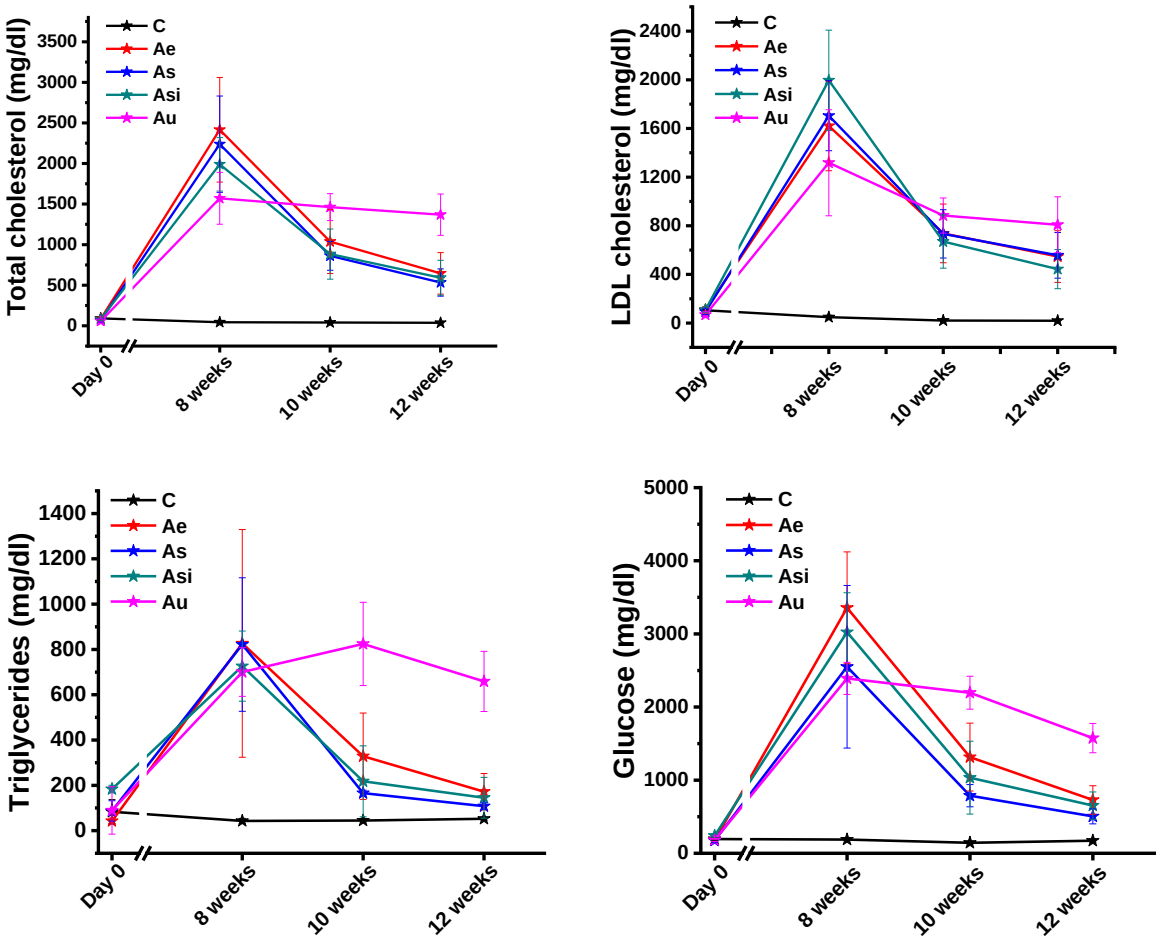

Figure 3: *Plasma quantitation of total cholesterol, LDL cholesterol, triglycerides and glucose levels for the 5 animal groups proposed in this study during the 12 weeks of animal experimentation: C- control group, Ae – early atherosclerotic group, As – statin and PCSK9 antibody stabilized atherosclerotic group, Asi – statin and PCSK9 siRNA stabilized atherosclerotic group and Au – vulnerable atherosclerotic group.*

Representative frozen sections of coronary arteries were laser- microdissected using the Zeiss PALM microbeam laser microdissection system (Oberkochen, Germany), to specifically isolate the coronary atheroma plaques (Fig. 4). The samples were suitably prepared for nano liquid chromatography – tandem mass spectrometric analysis using the Easy nLC II system coupled to the LTQ Orbitrap Velos Pro ETD hybrid mass spectrometer (Thermo Scientific, Illinois, USA), as previously mentioned (1). In parallel, tissue homogenates from carotid and ascending thoracic artery areas were performed to further corroborate the alarmin and other significantly altered protein levels along the lesion-prone area of the vascular tree. Sera samples harvested at the end of animal experimentation period were used for exosome isolation (using the miRCURY Exosome Serum/Plasma Kit from Qiagen, Venlo, The Netherlands) and proteomic analysis.

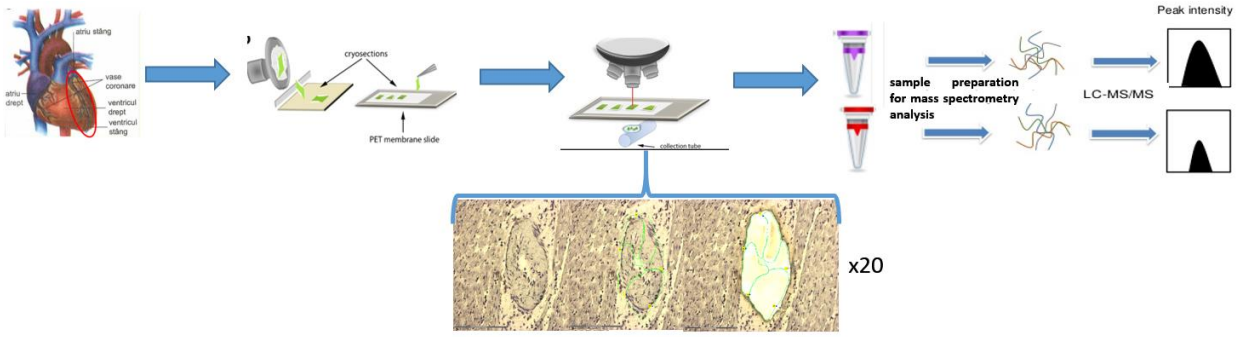

***Figure 4**: Coronary atheroma plaque laser microdissection workflow. Frozen coronary arteries, embedded in NEG-50 optimal cutting temperature medium were sectioned and stained with hematoxylin dye. The atherosclerotic lesions were laser-microdissected and the harvested material (~5mm^2) from each animal was solubilized and prepared for mass spectrometric analysis.*

The shotgun proteomic analysis (Proteome Discoverer 2.4, Thermo Scientific) revealed a plethora of alarmins and other molecules that were generally identified with high confidence in all animal groups proposed in this study.
The proteomic analysis of the microdissected atheroma plaque samples evidenced a total of 615 proteins, 77 of which were uniquely observed in the Ae group, 61 in the As group, 130 in the Asi group and 131 were only identified in the Au group. 41 proteins were common to all groups taken in this study (Fig. 5).

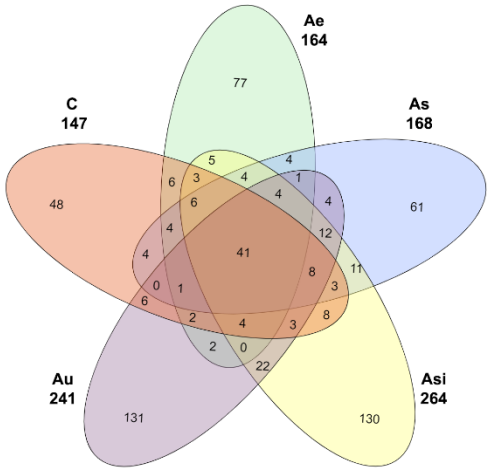

***Figure 5:** Venn intersection of the identified proteins from the laser microdissected atheroma plaques of the C, Ae, As, Asi, and Au animal groups.*

The alarmins identified in the experimental animal groups in all conditions and different areas of vascular bed are presented in Table 1. The recently performed protein inference will be closely followed by label-free relative mass spectrometric quantification and immunological validation. These studies will reveal which alarmins as well as the associated molecules will be differentially regulated by the hyperlipidemic diet and/or the anti-atherosclerotic treatment.

***Table 1**: Alarmins identified in the thoracic aorta, carotid, coronary artery samples and serum exozomes from all types of experimental animal groups (C, Ae, As, Asi, and Au) using mass spectrometric analysis. The experiment was performed in three technical replicates using three biological animals per group.*

The Sequest score is presented and reveals the confidence level of the identified proteins. A Sequest Score > 10 is generally accepted as the minimum confidence threshold of an inferred protein molecule.

No.	UniProt code	Protein description	Thoracic aorta	Carotid artery	Coronary artery	Serum exosomes
			Sequest score			
1	P51662	Annexin A1	7700.9	10244.48	-	-
2	P33477	Annexin A11	1544.57	1157.06	-	-
3	P15253	Calreticulin	4777.38	1282.5	-	-
4	O18750	Endoplasmin	3764.99	2842.18	-	-
5	Q28749	Fibronectin	1417.83	712.43	91.82	1471.68
6	P47845	Galectin-3	932.81	632.08	-	-
7	P30946	Heat shock protein HSP 90-alpha	5354.57	4002.75	-	-
8	P30947	Heat shock protein HSP 90-beta	4210.73	2350.49	-	19.35
9	Q6SQH4	Protein S100-A10	1130.59	345.2	-	-
10	P24480	Protein S100-A11	1765.33	1234.73	-	-
11	P34032	Thymosin beta-4	2549.04	1814.28	-	-

Using STRING bioinformatic platform, we revealed several over-represented Kyoto Encyclopedia of Genes and Genomes signaling pathways (Table 2).

Table 2: Kyoto Encyclopedia of Genes and Genomes over-represented signaling pathways by alarmins identified in the study.

KEGG signaling pathway	FDR p-value
Fluid shear stress and atherosclerosis	0.0027
IL-17 signaling pathway	0.0027
Cholesterol metabolism	0.0040
PI3K-Akt signaling pathway	0.0075

References:

1. Uyy, E. *et al.* Endoplasmic Reticulum Chaperones Are Potential Active Factors in Thyroid Tumorigenesis. *J. Proteome Res.* **15**, (2016).

The complex biochemical and mass spectrometry analysis are now in full progress. Aorta, coronary and carotid vascular fragments will be processed following the work flow shown in Fig.1. In parallel blood samples will be also analysed and the best candidates for molecular biomarkers will be validated by alternative methods (immunological, qPCR, immunohistochemistry). At least two scientific papers were in preparation to be submitted to peer review journals in the field.

2. Transnational collaboration

Describe the added value and synergies in the collaboration, any obstacles to the transnational collaboration, and the proposed solution (if necessary) (Max. 1 page).

The partner P2 appreciate very much the discussion we had with the CO in establishing the experimental model, in timing of harvesting the samples and the treatment delivery. The useful exchanging of ideas and significant references through the emails and regular skip meetings contribute to the adequate development of the project.

QUESTIONNAIRE / PROJECT IMPACT ASSESSEMENT

This questionnaire, to be filled by the project's coordinator, is part of the 2nd year's progress report (and is also included in the final report). It is for internal use by the ERA-NET ERA-CVD consortium in order to assess funded projects outputs. The results of this questionnaire will be presented in network meetings and included in public reports.

A. COMPOSITION OF THE CONSORTIUM

1. Please indicate the number and type of **entities** composing the consortium.

Type of entities	Total N°
Academia	2
Company	1
Clinical partner	1
Patient organisation	-
Other (Please specify: ……)	-

Add lines as appropriate

2. Please indicate the composition of the consortium in terms of gender balance.

Partner P1: ICBP N Simionescu	Total N° : 8
Females	6
Males	2

B. EXPECTATIONS VERSUS ACHIEVEMENTS

For each of the following areas, please rate to which extent (high, medium, low) you feel your project was expected to contribute and to what extent it has been contributing:

	Expected			Achieved		
	High	Medium	Low	High	Medium	Low
Scientific knowledge in some specific field	x			x		
Applied research and/or proof of concept	x			x		
Translational Research		x			x	
Impact on public health	x				x	
Research networking	x			x		
Training and capacity building	x			x		
Other (Please specify: new job creation)	x			x		

C. SCIENTIFIC KNOWLEDGE - PUBLICATIONS AND COMMUNICATIONS (after starting the project)

In the following tables, please list the **joint publications** and **communications** (in which ERA-CVD support was acknowledged) that resulted from the funded project (with at least two partners involved), underlining the name of the partners. In column "Partner(s) involved", please point out the project partners involved by using the numbering employed in the project proposal (e.g. partner 1 or P1).

1. Master and PhD theses

Master/PhD	Authors, title, year	Partner(s) involved
Master degree (2018-2020)	Aurel CERVEANU-HOGAS, Proteomic changes in carotid arteries induced by a hyperlipidaemia diet in New Zeeland White Rabbits, 2020	**P1**

Add lines as appropriate

2. Communications in scientific congresses

Authors, title, meeting name & place, year	Partner(s) involved	Type of presentation (Oral Communication/ Poster)
- Felicia Antohe. Toward precision medicine on the road from cell receptors to cell proteomics. The 40th ICBP NS Anniversary Symposium, Bucharest, 19-20 September 2019.	P1	Oral Communication
- Elena Uyy, Luminita Ivan, Viorel-Iulian Suica, Raluca Boteanu, Rune Hansen, Felicia Antohe. Alarmins expression in atherosclerotic lesions under severe anti-hyperlipidemic treatment; a mass spectrometry based proteomic approach. The 40th ICBP NS Anniversary Symposium, Bucharest, 19-20 September 2019.*	P1,CO	Poster
- Aurel Cerveanu-Hogas, Elena Uyy, Luminita Ivan, Viorel-Iulian Suica, Raluca Boteanu, Rune Hansen, Felicia Antohe. Mass spectrometric analysis of thoracic aorta alarmin profile in experimental atherosclerosis. 13th Central and Eastern European Proteomic Conference (CEEPC) Ustron, Poland, 23-25.09.2019.*	P1, CO	Oral Communication

-Viorel-Iulian Suica, Raluca Maria Boteanu, Elena Uyy, Luminita Ivan, Aurel Cerveanu-Hogas, Rune Hansen and Felicia Antohe. The proteomics of exosome alarmins as critical inflammatory players in coronary artery diseases.* 33rd World Congress on Cardiology and Heart Diseases, Prague, Czech Republic, 27-28.03.2020, (canceled event because of COVID 19).	**P1, CO**	**Poster**

D. USE OF SHARED RESOURCES/FACILITIES

1. Please indicate the number and designation of **shared resources** used within the framework of the current project.

Type of resources	Designation	Total N°
Existing cohorts		
Existing registries		
Biobanks		
Existing patient databases & corresponding biological material		
Innovative and shared technologies (e.g. OMICS, new generation	High performance LC/MS/MS tandem mass spectrometry proteomics	
Cellular and/or animal models	Experimental atherosclerotic New Zeeland White Rabbit model	40 males
Others (Please specify: ……)		

Add lines as appropriate

E. PUBLIC HEALTH IMPACT

1. Is the project expected to produce any result or finding that can be translated into a real impact on human health? YES

The project generated a list of alarmins highly expressed in the lesion in different stages of development that will represent a valuable asset for elaboration of clinical strategies for early detection and treatment of atherosclerosis. The best biomarkers will be selected to be used with the new multi-frequency ultrasound technology for improved evaluation and diagnosis of coronary plaque.

2. Has the project already produced any result or finding that can be translated into a real impact on human health? YES

Direct demonstration of proteome changes of the atherosclerotic lesions in different stages of development of the diseases that will be included in ProteomeXchange Consortium via the PRIDE partner repository data sets, free to be used by anyone.

3. If the answer to the questions G. 1 and/or G.2 was "YES", please indicate in what field(s) the results generated within the framework of the current project are expected to impact?

- Prevention
- Prognosis x
- Diagnosis x
- Therapy

4. Please list the major achievements of the consortium that can be translated into real impact on human health.

Achievements	YES/NO	Please specify (if YES)
Development of new preventive guidelines		
Development of new prognostic measures		
Development of new diagnostic products	Yes	List of alarmins up or down regulated in atherosclerosis, to be deposited in ProteomeXchange Consortium via the PRIDE partner repository international data sets.
Development of new diagnostic techniques	yes	Multi-frequency ultrasound technology for improved evaluation and diagnosis of coronary plaque.
Development of new diagnostic guidelines		
Development of new therapeutic products	yes	New alarmin biomarkers related to plaque progression
Development of new therapeutic techniques		
Development of new therapeutic guidelines		
Other achievements		

Add lines as appropriate

F. JOB CREATION

1. Please list below the number of **jobs** created within the framework of the current project (Pos-Doc fellowships & Contracts)

Partner #		Name	Gender	

	Type (Post-doc fellowship/ Contract)		**F**	**M**	**Duration of the fellowship/contract** months/years
ICBP NS	Master thesis and Research Assistant Contract	CERVEANU-HOGAS Aurel		x	Contract for at least 3 years

Add lines as appropriate

G. DISSEMINATION TO THE GENERAL PUBLIC

1. Which of the channels below were used to disseminate the results of the project to non-academic audiences and general public?

Dissemination channels	**YES/NO**	**Please specify (if YES)**
Project website	Yes	http://www.icbp.ro/static/en/en-networking_grants-grants-international/xplorecad.html
Presentations in non-scientific events, open days, etc.	Yes	Open access days of ICBP NS organised every year for young students to visit the research laboratories.
TV, Radio, Magazines, newspapers		-
Social Networks (twitter, linkedin, facebook, etc)		-
Internet (posting project news and communications in websites)	Yes	www.icbp.ro , P1 web site
Other channels		Central and Eastern European Proteomic Conference (CEEPC).

H. PARTNERSHIP AND CONSORTIUM SUSTAINABILITY

1. Did the partners of this project collaborate before applying for ERA-CVD joint transnational call (JTC)? YES

►If YES, please specify which partners (name, institution, country) were collaborating before and list the funding received (if applicable).

SINTEF (CO) and ICBP N.Simionescu (P1) collaborated previously in the MULTIBUBBLE (RCN FRINATEK, 2015-2018) project aiming to develop a new platform based on novel multifunctional nanoparticle-stabilized microbubbles and ultrasound technology for tergeted therapy of cancer and atherosclerosis.

2. Did you find any partners via the ERA-CVD Partnering Portal? YES/NO

►If YES, please specify which partners (name, institution, country) were found via the ERA-CVD Partnering Portal.

3. During the lifetime of the project, was there any new collaboration between any of the project participants and groups outside the consortium? YES/NO

►If YES, please specify which partners (name of researcher, institution, country) established new collaborations and with which groups (including other project consortia granted by ERA-CVD).

4. Has the participation in the project resulted in new scientific collaborations or partnerships between two or more of the project participants? YES

►If YES, please specify the partners (name, institution, country) involved.

The present collaboartion resulted in a new scientific research project with title: ***'A novel ultrasound-based therapeutic platform to enhance delivery of ApoA-1 encoding plasmid DNA for cardiovascular protection'*** by subitting a new applicationtion to The Norway Grants Call for Proposals 2019-Collaborative Reseach Projects with two Romanian partners and three colaborators from Norway:

CO: Felicia ANTOHE from Institute of Cellular Biology and Pathology N. Simionescu, Bucharest, Romania; **P1**: Runa HANSEN, from SINTEF AS, Trondheim, Norway; **P2**: Stefana PETRESCU, Biochemistry Institute, Bucharest, Romania; **P3**:Per SONTUM, Phoenix Solutions AS, Oslo, Norway; **P4**: Angelsen Bjørn, Surf Technology AS,Trondheim, Norway.

The project aim is, to improve the transfection rate of pDNA in the hepatocytes of the liver in order to obtain a systemic effect by elevating serum apoA-1 concentrations, affecting the cholesterol efflux and reducing the atherosclerotic burden, by combining ultrasound, microbubbles and liposomal pDNA.