DEPARTMENT OF MOLECULAR AND BIOMEDICAL SCIENCES

The research program of the Department of Molecular and Biomedical Sciences is focused mainly on basic research in protein biochemistry, molecular and cellular biology, and genetics. The primary goal of our investigations is the acquisition of new understanding of mammalian pathophysiology with the aim of improving human and animal health.

Toxinology

One of our traditional research topics in the field of toxinology is the study of molecular mechanisms of toxic action of secreted phospholipases A_2 ($sPLA_2$s) from animal venoms. In particular, we are focused on those endowed with presynaptic neurotoxicity (β-neurotoxins). The knowledge that we are gaining by studying toxic $sPLA_2$s is helping us to discover the pathophysiological roles of orthologous mammalian $sPLA_2$s, for example, their role in the development of neurodegenerative diseases such as Alzheimer's disease (AD).

In a proof-of-concept study, we showed that the rat group IIA $sPLA_2$ (GIIA) acted on the rat neuronal mitochondria in the same way as the snake venom β-neurotoxic GIIA. Moreover, the rat GIIA inhibited the activity of the cytochrome c oxidase (CCOX) also *ex vivo*, in the rat's brain tissue sections (Figure 1), additionally supporting the engagement of extracellular excessive GIIA in neurodegeneration, such as AD, by similar molecular mechanism as observed with the snake venom β-neurotoxic GIIA at its poisoning of the motoneuron (A. Ivanušec et al., *Int. J. Mol. Sci.* 23 (2022), 12368).

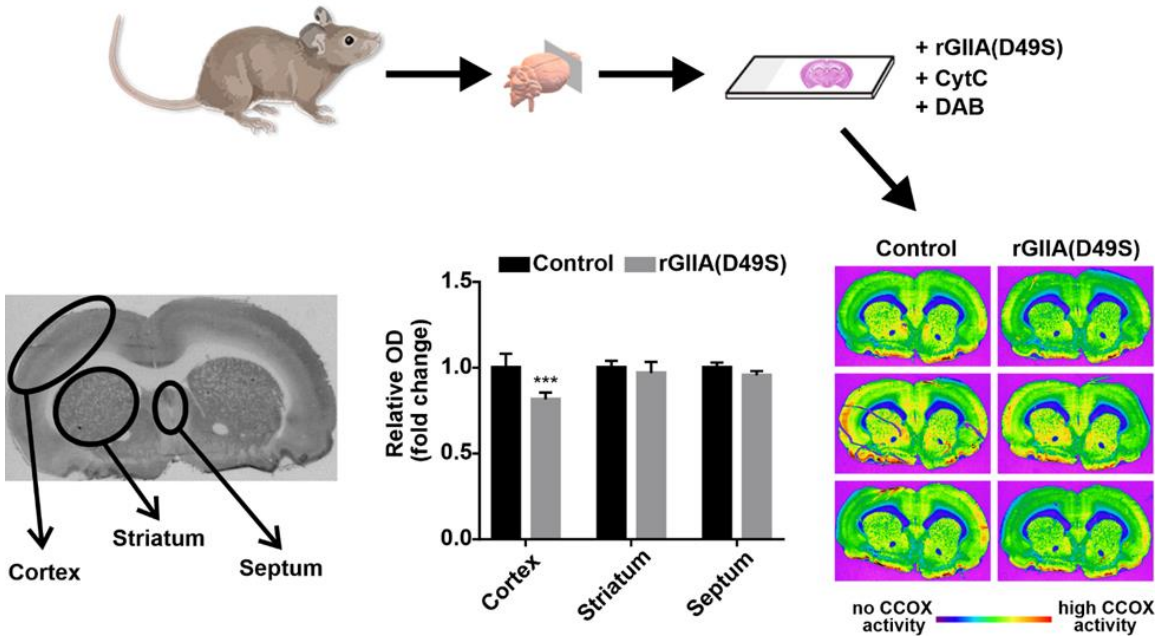

Figure 1: ***Ex vivo experiment on the rat brain tissue sections showed slight but significant inhibition of the CCOX activity in cerebral cortex in the presence of rat GIIA protein****. A trend towards decreased CCOX activity was detected also in the striatum and septum. Tissue sections were histochemically stained for CCOX activity in the absence or presence of rat GIIA protein, imaged and analysed for the relative optical density (OD) corresponding to the CCOX activity in different brain regions of interest.*

We monitored the intracellular trafficking of derivatives of recombinant ammodytoxin (Atx), rat GIIA and their enzymatically inactive (D49S) mutants in PC12 cells by transmission electron and fluorescence confocal microscopy, and concluded that $sPLA_2$ molecules do not require enzymatic activity to enter and traffic within the cell, including entering the mitochondria (A. Ivanušec et al., *Toxins* 14 (2022), 375). Using ^{125}I-GIIA, we determined the binding affinity of GIIA to a mitochondrial protein with an apparent molecular mass of 20 kDa (R20). Given that GIIA, like Atx, inhibits CCOX activity, we hypothesize that R20 is a subunit of CCOX IV (CCOX-IV). As we were not able to confirm this with the help of anti-CCOX-IV antibodies, we approached to identify R20 following its isolation from porcine mitochondria by the means of GIIA-affinity chromatography. We have been optimizing conditions for the isolation of the receptor. A precise description of the action of GIIA on CCOX is crucial for the use of our findings in medicine, both for the treatment of AD as well as for an early diagnosis of this severe neurodegenerative disease. Namely, it has been shown that GIIA in AD is overexpressed and becomes toxic to mitochondria, which is similar to the effects observed in Atx poisoned nerve endings.

It has been demonstrated that certain $sPLA_2$s specifically bind to nicotinic acetylcholine receptors (nAChRs). The binding of ACh or other agonists, such as nicotine and its derivatives, to nAChRs has been linked to uncontrolled cell division, prevention of apoptosis and induction of angiogenesis, ultimately supporting tumour growth and metastasis. Antagonists of nAChRs, however, showed opposite effects on cells, suggesting their potential value in cancer therapy. Among naturally occurring nAChR antagonists, found in various venoms, also snake venom $sPLA_2$s were shown to suppress ACh-elicited ion currents. For this reason, we investigated the anti-cancer effect of an array of human $sPLA_2$s and their single point enzymatically inactive mutants to assess their lung cancer therapeutic potential. In collaboration with pharmacologists from the University of Leuven, Belgium, we have been determining the effect of these proteins on α7 and muscle-type nAChRs. The most interesting result was obtained with GV(H48Q), which was selective for α7-nAChR. We used then GV(H48Q) to assess its effects on viability, cytotoxicity, proliferation and apoptosis of various lung cancer cell lines as well as one non-cancerous lung cell line. We demonstrated that GV and GV(H48Q) are able to prevent the ACh-induced cell proliferation and viability. In parallel, we were also involved in a similar study with another group of α7-nAChR antagonists, 3-alkylpyridinium salts (APS). The paper describing the effects of APS7, either free or packed in gelatine nanoparticles, on human lung cancer cells is almost finished (V. Kononenko et al., in preparation). In this area of our research, we also prepared two review papers, one has already been published (V. Kononenko et al., *Acta Biol. Slov.* 65 (2022), 5–17), the other is just to be submitted (T. Bele et al., in preparation).

Linked to $sPLA_2$s, we published a survey on pathophysiological actions of $sPLA_2$ molecules due to their binding to protein receptors – i.e. acting as ligands and not as enzymes, a scarcely studied area of increasing significance (A. Ivanušec et al., *Int. J. Biol. Sci.* 18 (2022), 873–888).

In 2022, we continued the study of snake venom proteins that affect the process of blood coagulation – haemostasis. In the scope of the research project J1-2475, funded by the Slovenian Research Agency (ARRS), we have been investigating a unique anticoagulant homologue of a serine protease from the venom of the nose-horned viper (*Vipera a. ammodytes*, *Vaa*), VaaSPH-1, in direction of developing completely new and safe drugs with anticoagulant activity. We searched for the best possible conditions for the expression of VaaSPH-1 as well as its binding protein, blood coagulation factor VIIIa (FVIIIa), in mammalian HEK293-F cells. In parallel, we have been designing low-molecular-mass FIX antagonists. We have a promising peptide candidate to test its action *in vitro*. On this subject, we wrote an invited review article on serine pseudoproteases (N. Zupanič et al., *FEBS J*. (2022), doi: 10.1111/febs.16355), exposing and discussing a profoundly neglected possibility of nonenzymatic functions of these SP molecules (Figure 2).

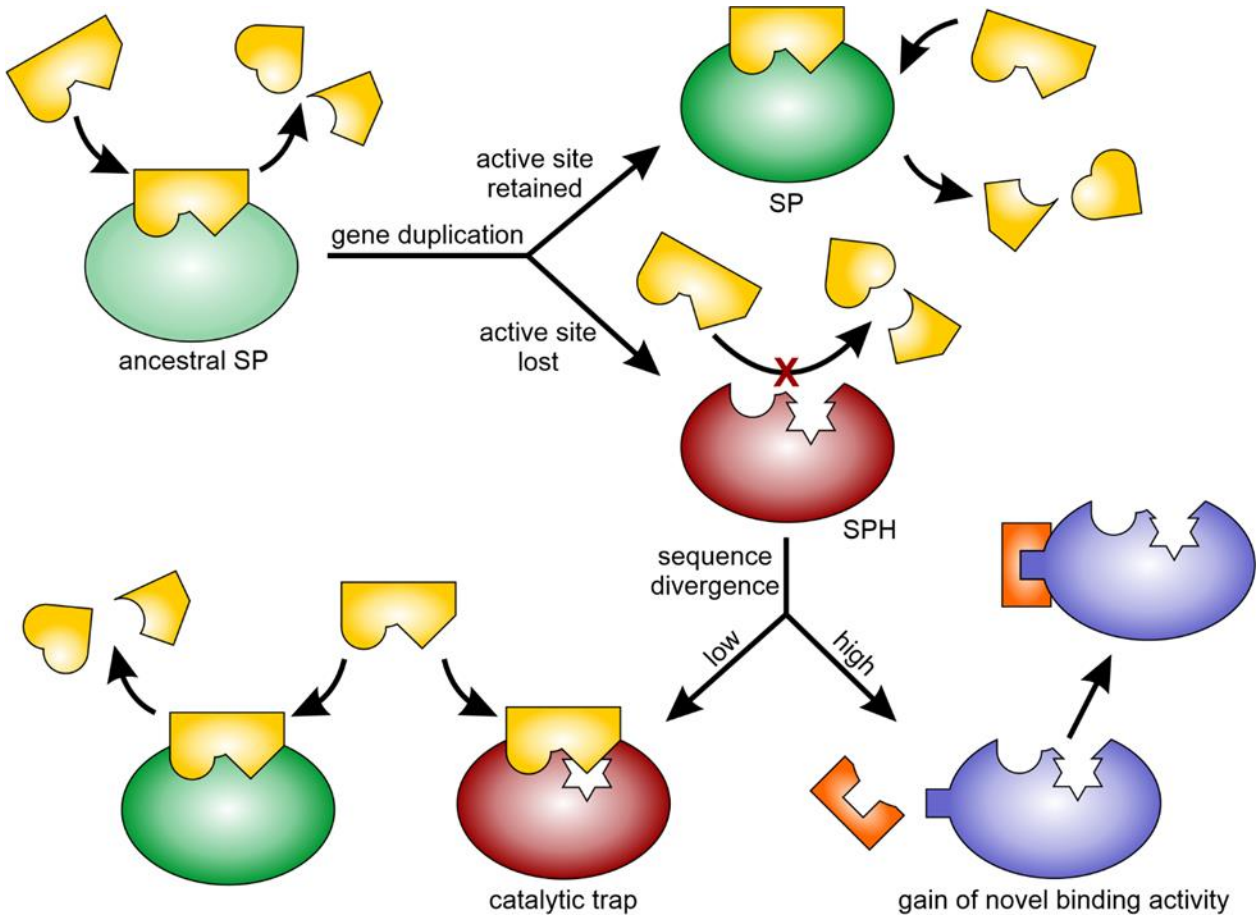

Figure 2: ***Evolution of novel biological functions in serine pseudoproteases****. Duplication of ancestral serine protease (SP) gene was followed by loss of catalytic activity in SP homologues (SPHs) due to mutations in the active site. The gain of novel functions (e.g. novel binding interface) in SPHs is the consequence of the specific pattern of amino acid substitutions. SPHs with low sequence divergence from their enzymatically active paralogues can no longer cleave polypeptide chains, but some of them can still bind to these chains and act as 'catalytic traps'. By retaining their binding to substrates, SPHs affect the function of cognate SPs by reducing the availability of substrate to be processed. The figure is from N. Zupanič et al., FEBS J. (2022), doi: 10.1111/febs.16355.*

After publishing a detailed description of the serine protease VaaSP-VX, which promotes blood clotting by activating both FV and FX, we isolated structurally very similar VaaSP-6 molecule from the *Vaa* venom. Since the entire cDNA sequence of VaaSP-6 is known, we will produce this protein recombinantly to characterize it. Hopefully, the recombinant VaaSP-6 will exhibit the same unique procoagulant activity as VaaSP-VX, so it could replace the unreliable dilute Russell's viper venom (dRVV) that is currently used in clinics for determination of lupus anticoagulants (LA test).

We have completed and published an extensive genetic, biochemical and physiological characterization of the VaaMPIII-3 protein from the nose-horned viper venom (K. Požek et al., *Toxins* 14 (2022), 232). By analysing its gene structure, we have unequivocally proved its structural exclusivity, and we proposed the introduction of a new subclass of metalloproteinases from snake venoms, subclass P-IIIe. For her Master's degree work dedicated to VaaMPIII-3, the first P-IIIe subclass protein, Kity Požek received the Krka Award (Figure 3) and the University of Ljubljana Prešeren Award.

Figure 3: ***Kity Požek with the Krka Award in the company of her co-mentor Dr Adrijana Leonardi at the 52nd Krka Awards ceremony****. Kity Požek received the Krka Award for her Master's degree work on a detailed description of VaaMPIII-3, the first member of the P-IIIe subclass proteins. For the same work, she also received the University of Ljubljana Prešeren Award.*

Within our large international project on the *Vaa* genome sequencing and analysis with the Technical University of Denmark and Beijing Genomics Institute, we estimated that the size of the *Vaa* genome is about 1.61 Gb. More than 47% of the nose-horned viper genome has been annotated as transposable elements, and close to 22,000 protein-coding genes have been predicted.

Together with colleagues from the Centre for Clinical Toxicology and Pharmacology, University Medical Centre Ljubljana (UMCL), we have investigated an interesting clinical effect in patients envenomed by the nose-horned viper, namely a profound, transient and reversible thrombocytopenia of functional platelets, as part of the ARRS research project J3-2534. In thromboembolic diseases, such as myocardial infarction and ischemic stroke, platelets play a central role. Existing antiplatelet drugs have one common side effect – a reduced number of platelets whose activity is inhibited. This condition carries a high risk of bleeding (haemorrhage), especially in interventional cardiology and angiology that use an antithrombotic approach. Our findings may pave the way for the development of a new group of antiplatelet agents that would reduce the risk of dangerous bleeding in interventional cardiology and angiology, and increase the efficacy of vasodilation and clot removal. We demonstrated that the reversible thrombocytopenia in patients envenomed by the *Vaa* is induced by proteins similar to type C lectins (snaclecs). In the past year, we isolated several snaclecs from the *Vaa* venom and showed that particularly snaclec 3/2 is a strong inducer of thrombocytopenia through its interaction with the GPIb platelet receptor. In collaboration with our partners from the Faculty of Veterinary Medicine, University of Ljubljana (VF/UL), we initiated an *in vivo* study in a mouse model of arterial thrombosis to validate the potential of snaclec 3/2 to prevent clot formation and arterial occlusion after experimentally induced vascular injury and to determine its potential for medical applications. Two papers on this topic are currently in preparation (M. Dobaja-Borak et al., in preparation; M. Žužek et al., in preparation).

Within the research network, comprising experts from UMCL, University Hospital and University of Split, University of Zagreb (UZ) and from our group, we analysed samples of patients who were envenomed by the *Vaa* and treated with different antidotes. A publication is underway (T. Kurtović et al., in preparation).

Lipid metabolism and signalling

Our research in the field of lipid metabolism and signalling is focused on the role of lipid droplets in inflammatory signalling, autophagy and ferroptosis. Our work was focused on the following fundamental questions in lipid biology: (1) What is the role of the lipid droplet organelle in fatty acid trafficking and the regulation of membrane oxidation?, (2) How does autophagy cooperate with lipolysis in lipid droplet breakdown during nutrient stress (Figure 4)? and (3) Are lipid droplets required for the production of lipid mediators of inflammation?

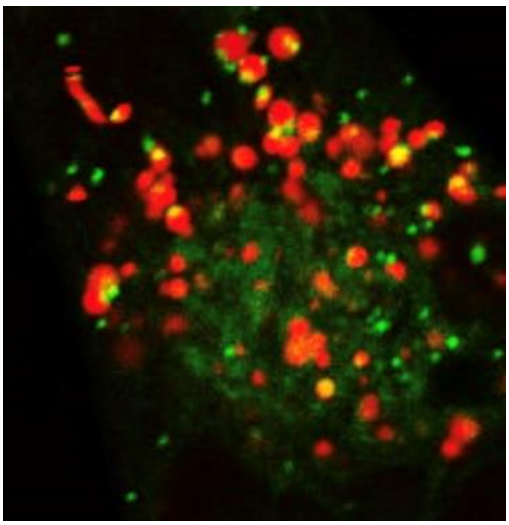

Figure 4: ***Colocalization between lipid droplets and autophagosomes in starving cancer cells****. Live-cell confocal microscopy imaging of lipid droplets stained with BODIPY 493/503 (green) and autophagosomal structures labelled with the marker protein LC3 fused with a red fluorescent protein (red). The overlapping signals (yellow) of the two organelles suggest that lipid droplets are broken down via autophagy. The image was taken by our PhD student Špela Koren.*

Cells carefully control the oxidation of lipids to balance desired oxidative conversion of polyunsaturated fatty acids into lipid signalling molecules with unwanted peroxidation reactions that lead to membrane dysfunction, inflammation and potentially cell death. Oxidized lysophospholipids are emerging as novel damage-associated molecular patterns that promote sterile inflammation and contribute to the pathology of chronic and aging-related diseases. In a review paper (T. Petan & M. Manček-Keber, *Free Radic. Biol. Med.* 188 (2022), 351–362), we gathered and critically evaluated the current knowledge on their biosynthesis and release from cells, the cellular processes that drive their formation as well as their (patho)physiological roles. Additionally, we discussed the potential use of phospholipase and oxidative enzyme inhibitors in the prevention of oxidized lysophospholipid formation, which might revive clinical research with existing inhibitors and foster the development of new strategies for treating inflammatory diseases.

In 2022, we completed our experimental work on the role of lipid droplets in the production of lipid signalling molecules. We were able to show that lipid droplets drive the production of mitogenic lipid mediators in cancer cells not only cell culture but also in xenograft tumor models. This work was recently submitted and is under review (E. Jarc Jovičić et al., *bioRxiv* (2022), 2021.11.25.470010). In a collaborative work with colleagues from the Biotechnical Faculty, University of Ljubljana (BF/UL), we studied the possible role of lipid droplets in the protection of endothelial cells from iron oxide nanoparticles (N. Repar et al., *Int. J. Mol. Sci.* 23 (2022), 6972). We found that the induction of lipid droplet biogenesis is not required for the ability of exogenous monounsaturated fatty acids, such as oleic acid, to protect from nanoparticle-induced stress. We also collaborated on a study focused on the subcellular trafficking of herpesviruses (M. Mavri et al., *Front. Endocrinol.* 13 (2022), 862940).

We presented our work at numerous international conferences and workshops, which included the invited talks at the 17th GERLI Lipidomics Meeting, Nice, France and the Training School in Lipid Metabolism, TU Dresden, Germany.

High-throughput genetics and functional genomics in yeast *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* is a well-established model organism for basic research and a cell factory in biotechnology. Recently, it has also become an important feature in synthetic biology for the homology recombination-based *in yeasto* assembly of DNA fragments. Following our development of techniques for polygenic trait analysis in yeast, we have started assembling a toolbox for hierarchical DNA assembly by combining *in vitro* and *in yeasto* approaches.

Within the ARRS project L4-3181, *Hierarchical DNA assembly for advanced applications in biopharmaceuticals production and cell therapy*, we developed a toolbox for the assembly of

combinatorial variants of plasmids and smaller genomes. This approach can also be combined with multiplex CRISPR-Cas systems for further genome editing. We published a research paper in which we describe our study to evaluate precise targeting of multiple loci simultaneously using multiplex CRISPR-Cas9 (G. Žun et al., *Yeast* (2023), doi: 10.1002/yea.3833; accepted in 2022). One of the features of this work is a CRISPR-Cas9 system that enables simultaneous edits of up to 5 genomic loci (Figure 5).

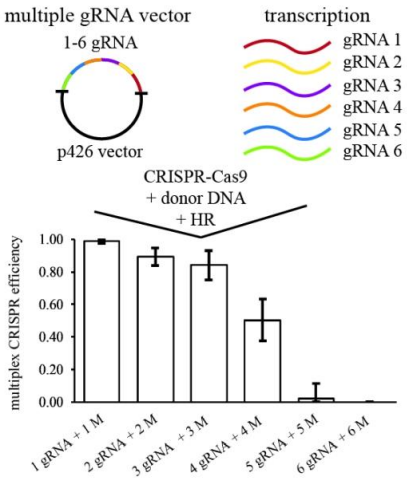

Figure 5: ***Multiplex CRISPR-Cas9 for up to five successful simultaneous genome edits****. Constructs to express gRNAs individually were assembled as BioBricks and then the evaluation of single and multiplex CRISPR-Cas9 gRNA systems was performed (G. Žun et al., Yeast (2023), doi: 10.1002/yea.3833).*

In addition to multiplex CRISPR-Cas9, we started developing in 2022 methods for epigenome engineering and for genome editing whereby specific cellular DNA repair mechanisms are selected for. Our expertise in genome editing with CRISPR-Cas9 methods featured also in a collaborative research project lead by Dr Gianni Liti (CNRS, France) which resulted in a joint publication entitled Domestication reprogrammed the budding yeast life cycle, published in the prestigious journal *Nature Ecology & Evolution* (M. De Chiara et al., *Nat. Ecol. Evol*. 6 (2022), 448–460).

In the field of biotechnological research, we screened more than 1,400 non-conventional yeast strains for the ability to store high lipid amount from short-chain fatty acids as the main carbon source, and identified some potentially biotechnologically interesting strains. This work was done in collaboration with our partners in the ERACoBioTech project OLEOFERM (https://oleoferm.eu/).

Natural habitats are the most important source of yeast strains with biotechnologically interesting traits and within this line of research we participated in a consortium of authors of a review paper Yeasts from temperate forests (S. Mozzachiodi et al., *Yeast* 39 (2022), 4–24), whereby we contributed to the part describing the isolation of biotechnologically promising yeast strains.

Evolutionary genomics

The proteus (*Proteus anguinus*) has exceptional morphological and physiological adaptations to the subterranean environment, with regenerative ability, high resistance to prolonged starvation, and a lifespan that may exceed 100 years. The international Proteus Genome Research Consortium (http://proteusgenome.com) has been established to tackle the challenge of sequencing the proteus genome and its transcriptomes. In the scope of ARRS project J1-2469, led by our colleagues at BF/UL, we have been participating with the analysis of genomic and transcriptomic data. The first paper on this subject provides the scientific and biomedical rationale for exploring the proteus genome and outlines potential outcomes, challenges, and methodological approaches required to analyze and annotate the genome of this unique amphibian (R. Kostanjšek et al., *Ann. NY Acad. Sci.* 1507 (2022), 5–11). In the middle of 2022,

the first draft assembly of the huge Proteus genome was made at the Beijing Genomics Institute, which is, with its 34 Gb, among the largest genomes ever sequenced, more than 10-fold larger than the human genome. Besides the analysis of transposable elements (TEs) we participate on the investigations of diverse cave adaptations, on the analysis of the chemosensory system of this blind animal, its G-protein-coupled receptors repertoire (GPCRome) as well as on the genome defense systems against TEs (*APOBEC*, *SCAN-ZNF* and *KRAB ZNF* genes). With the genome data, we also updated some of our previous findings based on the transcriptome data (*e.g.* olfactory receptors, V1R and V2R vomeronasal receptors and taste receptors).

We also performed a comprehensive analysis of the papain superfamily of cysteine peptidases, using the extensive proteomic, transcriptomic and genomic data for Archaea, Bacteria and Eukaryota. It has provided new insights into their origin, evolution and classification. A publication is underway (D. Kordiš & V. Turk, in preparation).

Other subjects

In 2022, we also participated in different projects out of the thematic framework of our department, funded by ARRS or other funders. Mentioned below are only those projects, on which publications have been already prepared.

In the scope of the ARRS project J1-2482 (leading institution: BF/UL), we have been determining the impact of environmentally relevant nano- and microplastics on terrestrial vertebrates by mass spectroscopy. We also performed the proteomic analysis of the haemolymph of the terrestrial crustacean *Porcellio scaber* and revealed components of its innate immunity under baseline conditions (A. Jemec Kokalj et al., submitted).

As partners on the ARRS project J2-3040 on magnetically controllable nanocarriers that mimic endogenous lipid particles to improve drug/nanoparticle delivery, we participated at analysing the effects of barium-hexaferrite nanoplatelets in low-frequency magnetic field on cancer cells (T. Goršak et al., in preparation).

We also collaborated informally with several groups at home and abroad. Colleagues from the Ruđer Bošković Institute and UZ were assisted in researching the mechanism of formation and morphogenesis of biomineral nanostructures of the *Archa noae* shell. We performed structural identification of protein components of the shell that are potentially involved in the biomineralization process (I. Sondi et al., in preparation).

In the study led by colleagues from the Faculty of Electrical Engineering UL (FE/UL), we analysed the protein corona composition of nanoparticles using a proteomic approach to explain their toxic impact on the human immune system (M. Pavlin et al., *Int. J. Mol. Sci.* 23 (2022), 6197).

Invited by the Editor-in-Chief of *Acta Biologica Slovenica*, a review paper on mass spectrometry in snake venom research was prepared (A. Leonardi, *Acta Biol. Slov.* 65 (2022), 5–25).

We came to the aid to our colleagues from MF/UL performing the confocal microscopic analysis in functional validation of an α-FREM2 nanobody as molecular tool to target specifically glioblastoma stem cells (N. Šamec et al., submitted). In the same area, we joined our colleagues from the National Institute of Biology in Ljubljana to prepare an extensive analysis on bioactive peptides from venoms against glioma progression (B. Majc et al., *Front. Oncol.* 12 (2022), 965882).

Some outstanding publications in the past year

1) De Chiara, M., Barré, B.P., Persson, K., Irizar, A., Vischioni, C., Khaiwal, S., Stenberg, S., Chioma Amadi, O., Žun, G., Doberšek, K., Taccioli, C., Schacherer, J., Petrovič, U., Warringer, J. and Liti, G.: Domestication reprogrammed the budding yeast life cycle. Nature Ecology & Evolution, 6 (2022), 448–460

2) Požek, K., Leonardi, A., Pungerčar, J., Rao, W., Gao, Z., Liu, S., Laustsen, A.H., Trampuš Bakija, A., Reberšek, K., Podgornik, H. and Križaj, I.: Genomic confirmation of the P-IIIe subclass

of snake venom metalloproteinases and characterization of its first member, a disintegrin-like/cysteine-rich protein. Toxins, 14 (2022), 232
3) Ivanušec, A., Šribar, J., Leonardi, A., Zorović, M., Živin, M. and Križaj, I.: Rat group IIA secreted phospholipase A_2 binds to cytochrome c oxidase and inhibits its activity: A possible episode in the development of Alzheimer's disease. International Journal of Molecular Sciences, 23 (2022), 12368
4) Zupanič, N., Počič, J., Leonardi, A., Šribar, J., Kordiš, D. and Križaj, I.: Serine pseudoproteases in physiology and disease. FEBS Journal, (2022), doi: 10.1111/febs.16355
5) Kostanjšek, R., Diderichsen, B., Recknagel, H., Gunde-Cimerman, N., Gostinčar, C., Fan, G., Kordiš, D., Trontelj, P., Jiang, H., Bolund L. and Luo, Y.: Towards the massive genome of *Proteus anguinus*, illuminating longevity, regeneration, convergent evolution and metabolic disorders. Annals of the New York Academy of Sciences, 1507 (2022), 5–11
6) Petan, T. and Manček-Keber, M.: Half is enough: Oxidized lysophospholipids as novel bioactive molecules. Free Radical Biology and Medicine, 188 (2022), 351–362